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A VARIANT OBTAINED DIRECTLY FROM PNEUMOCOCCUS TYPE II

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A great many observations have been made during the last quarter of a century on the dissociation of pneumococci and its relation to morphological and serological changes. Even as early as 1892, Kruse and Pansini showed that pneumococci might exist in two forms—one possessing little virulence, growing in chains and having no capsule; the other being lancet-shaped, well-encapsulated, and very virulent. Neufeld (1902) demonstrated that when virulent pneumococci are grown in immune serum they lose their virulence and their power to form capsules. Again, in 1905, Kindborg pointed out that there were two forms of pneumococci and at the same time proved that the less virulent of the two grows much better in artificial media.

More recently the studies of Stryker (1916); Blake and Trask (1923); Reimann (1925); and Amoss (1925) have definitely proved that when virulent strains of pneumococci are grown in media containing immune serum, in media containing bile, or in slightly acid media there will be produced striking changes in colony formation, type specificity, agglutinability, capsule formation and virulence.

Practically all of the results, reported by the above mentioned investigators, were obtained by the use of purely artificial media, or of such media modified by the addition of various substances, such as immune serum, acid, or bile.

The variant described in this paper was obtained not by the employment of artificial media, but from what is considered to be the best medium for cultivating pneumococci, namely the peritoneal fluid and heart blood of mice which had died from the

injection of minimal lethal doses of a very virulent strain of Pneumococcus Type II. This "stock strain" of pneumococcus was obtained from Dr. A. K. Poole of the Department of Clinical Medicine, and had been isolated from the blood stream of a patient suffering from pneumonia.

When the culture was obtained it was a Gram-positive lanceolate diplococcus possessing a well-defined capsule. It was bile soluble, fermented inulin, and agglutinated with Type II antiserum in high dilutions. On fresh rabbit blood agar plates the colonies, when observed under a magnification of 15 ×, were slightly raised, smooth, with the characteristic concave surface of pneumococcus colonies. Growth on this medium was always accompanied by a reduction of hemoglobin in the immediate vicinity of the colony. The organism had, at this time, a moderate degree of virulence, approximately 20 cells killing a white mouse within twenty-four hours.

This particular strain was being utilized in a study designed to develop a bacteriophage for type II pneumococcus, the results of which will be published at a later date. In the course of this work two types of organisms were produced by the action of a lytic principle which had been derived from sputum. One of the pneumococcus types was extremely virulent—three organisms killing a mouse within 14 hours. It was a lanceolate diplococcus and grew better at 20°C. than at 37°C. The other assumed a bacillary form; was not virulent; and enjoyed a wide range of temperatures—from ice box to 37°C. It was found later in this work that an organism exactly similar to the one just mentioned (bacillary form) could be obtained by passing the "stock strain" through mice for the purpose of increasing and maintaining its virulence.

The original strain was transferred daily into fresh rabbit blood pneumococcus broth (5 drops of fresh blood in 10 cc. of beef infusion broth having a pH of 7.6 to 7.8). Every other day 0.05 cc. of this virulent culture was injected into the peritoneal cavity of a white mouse. The virulence of the "stock strain" was increased and maintained by this method so that five organisms when injected intraperitoneally would kill a mouse within eighteen

hours. Material from the peritoneal cavity and heart blood was examined directly by tinctorial methods and was also streaked on fresh blood agar plates. These plates were incubated for eighteen to twenty-four hours at 37°C. and the colonies were examined for any changes by the aid of a dissecting microscope having a magnification of 15 ×. Typical pneumococcus colonies were picked off the plate and transferred into fresh blood pneumococcus broth and incubated for the same length of time as the plates. The virulence of this strain never exceeded a dilution of 10^{-7} (approximately 5 organisms). However, when the maximum virulence of the original strain had been reached and was being maintained, during the examination of the typical colonies obtained from the peritoneal fluid and heart blood there appeared very small colonies near to, but never in contact with, the comparatively much larger typical pneumococcus colonies. These very small colonies appeared either singly or more commonly in groups of five arranged rosette fashion around the pneumococcus colonies.

These tiny, smooth colonies were picked off the plate under the microscope with the aid of an extremely fine platinum needle. When they were stained by the use of Gram stain or methylene blue no clear cut picture of any bacterial forms could be recognized. Instead, there appeared heavily stained, highly refractile spherical, triangular, or irregularly shaped bodies.

At the same time individual colonies were also inoculated directly into either blood pneumococcus broth or plain pneumococcus broth and incubated for eighteen to twenty-four hours at both 37° and 20°C. When these tubes were removed from the incubator at the end of this time and examined macroscopically no evidence of any growth appeared; the broth remaining as clear in the inoculated tubes as in the sterile broth control tubes. However, when the material from the several tubes was stained with the Gram stain, highly refractile bodies could be seen. These masses were quite large, spherical, or nearly spherical in shape, and there was a tendency for most of the individuals to remain well isolated, although it was not uncommon to see them in clusters. (See fig. 1.)

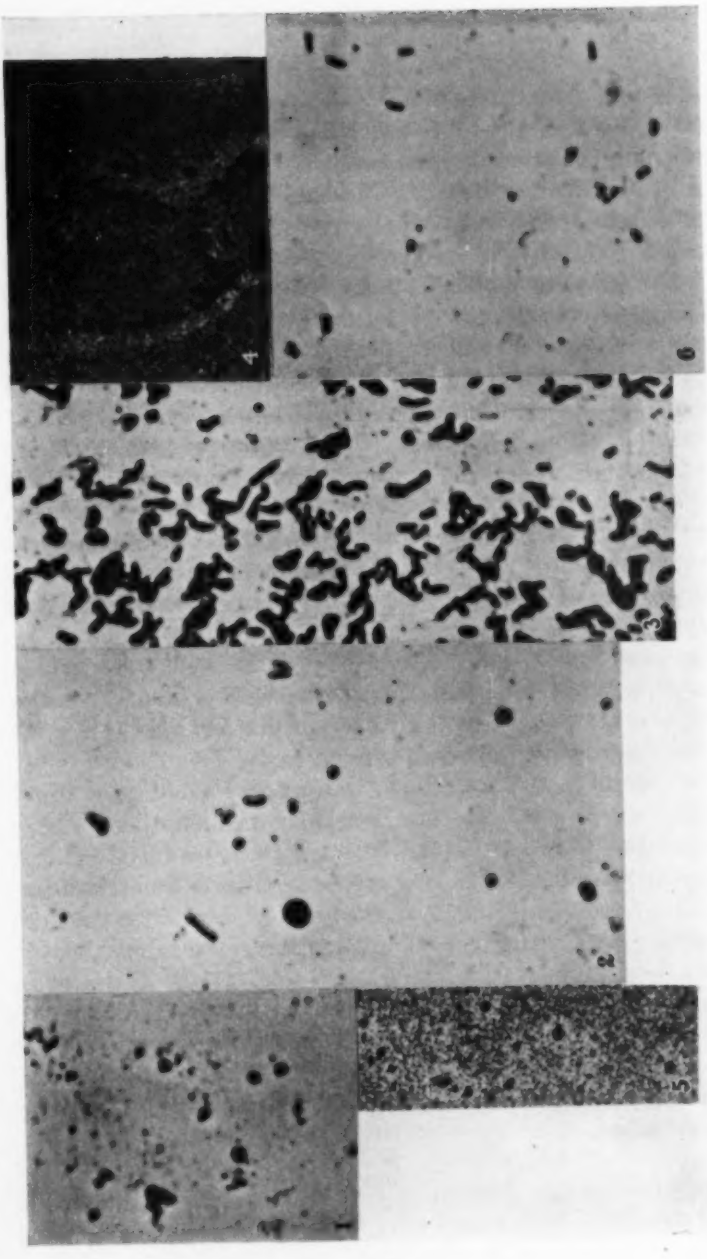
The tubes were returned to the incubators for another eighteen- to twenty-four-hour period of incubation, but before doing so, 0.05 cc. from each tube was placed on a fresh blood agar plate and streaked with sterile glass rods. These plates were also incubated at the same temperatures as the broth cultures from which the material was obtained.

Following this second incubation the broth cultures showed macroscopic evidence of a definite, extremely fine granular suspension in the clear broth. This granular substance appeared heaviest in the plain broth tubes, the blood pneumococcus broth supporting the growth of the variant only to a meagre extent. Upon examining stained preparations from the tubes, various shaped bodies could be made out. Some were still spherical, others were definitely curved, giving the impression of vibrios, while the remainder were either short thick rods, or longer, less thick, bacillary forms. (See fig. 2.)

The growth on the plates, although very luxuriant, was difficult to see due to the extremely small size of the colonies. It was only when the plates were held so that direct light struck them obliquely that the mass of colonies could be discovered. With the naked eye they seemed more like a part of the surface of the plate than distinct bacterial growth. However, when the plates were examined with the dissecting microscope the small, smooth colonies could be easily distinguished. It should be emphasized that if the plates were given only a customary glance for evidence of growth it would most likely be missed, to the naked eye the plates appearing to be absolutely devoid of growth. Stained preparations from individual colonies from these plates revealed essentially the same picture as that described above.

Again the tubes were returned to the incubators for another eighteen- to twenty-four-hour period, but before doing so fresh blood agar plates were seeded in the same fashion as before.

At the end of the seventy-two-hour incubation period the broth, instead of being crystal clear and supporting a fine granular suspension, was faintly turbid throughout. Stained preparations showed that the organisms were now definitely rod-shaped and strongly Gram-positive. Here and there, however, vibrio-like



Figs. 1-6

forms could be seen. (See fig. 3.) The growth on the blood agar plates seeded from the tubes which had been incubated for forty-eight hours consisted of very fine, small colonies. Around the mass of colonies the hemoglobin had been reduced, forming a fringe of hemolysis which could be made out very easily. Figure 4 shows a mass of these colonies photographed and magnified 10 ×. The fringe of hemolysis can be observed as well as several individual colonies. Figure 5 also shows individual colonies photographed and magnified 10 ×. The zone of hemolysis can easily be seen. When these colonies were examined by tinctorial methods the individuals were entirely Gram-positive bacilli.

Once the culture had attained the amount of growth evidenced by a faint turbidity, a small amount transferred into fresh broth gave equally good evidence of growth within twenty-four hours either at room temperature or at 37°C. When these subcultures (twenty-four hours) were stained they always presented a picture similar to that of figure 3. Subcultures on fresh blood agar plates always gave good evidence of growth after an equally long incubation period. However, if a culture is allowed to remain at room temperature for a period as long as 14 days and then examined one can see evidence of diplococcus-like forms as well as the bacillary type which predominates. (See fig. 6.)

The variant is very stable, and transplants made from cultures standing at room temperature for as long as a month grow very luxuriantly. The culture can be maintained on blood agar at ice-box temperature for as long as four months without transferring onto fresh media. Indeed, the first few days after the fresh culture is placed in the ice-box the colonies increase in size slightly. It is a Gram-positive bacillus; is bile soluble; and agglutinates with Type II antiserum up to a dilution of 1:400. It also agglutinates to a very slight extent with Type I antiserum. It has never been pathogenic for mice; as much as 2 cc. of a very heavy, fresh suspension has been injected into the peritoneal cavity without affect.

The parent strain of pneumococcus when originally obtained in its moderately virulent form never showed associated with it any

of these very small colonies. It was only when the maximum virulence of this strain of pneumococcus had been reached that the variant appeared. So long as the virulence of the culture was maintained at this high point, one could readily isolate the variant from the heart blood or peritoneal fluid of mice which had died from a minimal lethal dose of the pneumococcus. It is not known just when this change occurs, for when mice which were almost dead were killed and autopsied, the variant could be obtained from their body fluids as easily as from those which had died during the course of the infection.

This variant differs materially in morphology, pathogenicity, and temperature requirements from the original strain. However, it retains some features which still characterize it as a pneumococcus; bile solubility, ability to reduce hemoglobin, and agglutinability with type specific antiserum in fairly high dilutions. It is hoped that further work with this variant will cause it to return to a lanceolate diplococcus. Once that is achieved the question of restoring it to its original virulence can be pursued.

The fact that one can isolate under ordinary conditions, pneumococci from human throats suggests that many of the Gram-positive bacilli found in normal throats and so vaguely classified, may be products of pneumococci. As such they may play a very small rôle in the health of the individual harboring them. However, it is possible that certain conditions may lead to a reversion of these innocuous forms to virulent types, when they may become highly invasive.

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A SIMPLE MINERAL MIXTURE AGAR FOR THE CULTIVATION OF PASTEURELLA BOVISEPTICA[1]

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During the past few years many experiments have been made on the cultivation of anaerobes, especially *Clostridium chauvei* which are grown with difficulty in culture. It was found that ferric salts increased the virulence of cultures of this organism, while ammonium dihydrogen phosphate, sodium citrate, and potassium tellurite increased the production of aggressive substances without increasing virulence. These experiments led to the study of the action of 68 salts on the growth, gelatin liquefaction, and fermentation of anaerobic organisms.

The optimum concentration of the 68 salts was determined by growing *Cl. welchii*, *Cl. novyi*, and *Cl. sporogenes* in brain medium, broth, and gelatin containing 0.5 per cent peptone and 0.5, 0.05, 0.005, 0.0005, and 0.00005 molar concentrations of the salts. A study of the essential ions necessary for the growth of these anaerobic organisms and of *Cl. septicus* (*Cl. oedematis-maligni*) and *Cl. chauvei* was made by making a mixture containing all 20 ions found in the 68 salts and 19 mixtures, from each of which a single ion was omitted. Mixtures which caused a marked decrease in the growth of the five test organisms were considered to have an essential ion missing. Mixtures of ten ions which caused a decrease in growth were then made and again tested. This was repeated for seven ions and finally for six ions. It was found that only five of the 20 ions tested are essential for the growth of these anaerobic organisms. These essential ions are

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potassium, ammonium, dihydrogen phosphate, bicarbonate, and pyruvic acid.

It was found that these salts did not stimulate liquefaction of gelatin or fermentation as readily as other salts. Potassium chloride, potassium hydrogen phosphate, and sodium hydrogen phosphate were more efficient in causing liquef action of gelatin by *Cl. septicus*, *Cl. chauvei*, and *Cl. novyi*, than salts containing the essential ions. Similarly ferric ammonium citrate, magnesium sulfate, and disodium hydrogen phosphate increased the fermentation reactions of these organisms.

From this study it was thought possible that an agar medium could be developed which would grow *Pasteurella boviseptica* more readily than ordinarily infusion agar and it was also hoped that the virulence of the organisms could be more readily maintained.

The optimum concentration of potassium chloride, potassium bicarbonate, sodium chloride, ammonium dihydrogen phosphate, potassium citrate, and ferric ammonium citrate were made. An aqueous agar and a beef heart decoction agar containing 0.5 per cent peptone and 1.5 per cent Difco Bacto agar were prepared and adjusted to pH 7.2. Nine cubic centimeters of the media were distributed in test tubes which were then stoppered and sterilized at ten pounds pressure. The six salts were added so as to make final solutions of 0.5, 0.05, 0.005, 0.0005, and 0.00005 molar concentration. The tubes were then cooled in a slanting position. Inoculation was made from a saline suspension of a twenty-four hour growth of different strains of *Pasteurella boviseptica*, and the amount of growth was compared at the end of twenty-four and forty-eight hours. Table 1 shows the results obtained from the comparison of the growth of two strains of *Pasteurella boviseptica* in these mineralized agars.

It is seen that *Pasteurella boviseptica* grows quite readily in all dilutions of sodium and potassium chloride tested. The greatest differences in growth were found to occur in the case of potassium bicarbonate and ammonium dihydrogen phosphate.

To determine the growth promoting powers of these salts, 40 mixtures of them were made. Each mixture was added to beef heart infusion agar containing 1 to 2 per cent peptone, and 2 per

TABLE 1

Optimum concentration of salts

SALT	CONCENTRATION MOLAR	GROWTH*									OPTIMUM	
		Culture 2				Culture 5						
		Broth agar		Aqueous agar		Broth agar		Aqueous agar				
		24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	Total	Molar	Per cent
Sodium chloride	0.5	1	1	1	1	1	1	½	1	7½	0.05	0.29
	0.05	1	2	1	1	1	1	1	1	9		
	0.005	1	1	1	1	1	1	1	1	8		
	0.0005	1	1	1	1	1	1	1	1	8		
	0.00005	1	1	½	1	1	1	1	1	7½		
Potassium chloride	0.5	½	1	½	1	½	½	0	0	4	0.05	0.37
	0.05	½	2	1	2	½	1	½	1	8½		
	0.005	1	1	1	1	1	1	1	1	8		
	0.0005	1	1	1	1	1	1	½	1	7½		
	0.00005	1	1	1	1	½	1	1	1	7½		
Potassium bicarbonate	0.5	0	0	0	0	0	0	0	0	0	0.005	0.05
	0.05	1	1	1	1	0	0	0	0	4		
	0.005	2	2	1	1	2	2	2	2	14		
	0.0005	1	2	1	1	2	2	1	2	12		
	0.00005	1	2	1	1	2	2	1	1	11		
Potassium citrate	0.5	0	0	0	0	0	1	0	1	2	0.005	0.16
	0.05	1	1	1	1	1	2	1	2	10		
	0.005	1	2	1	1	2	2	2	2	13		
	0.0005	2	2	2	2	1	1	1	2	13		
	0.00005	1	1	1	1	2	2	2	2	12		
Ammonium dihydrogen phosphate	0.5	0	0	0	0	1	2	1	1	5	0.005	0.06
	0.05	1	1	1	1	1	2	1	2	10		
	0.005	2	2	1	2	2	2	2	2	15		
	0.0005	1	2	1	1	2	2	1	1	11		
	0.00005	2	2	1	1	1	1	1	1	10		
Ferric ammonium citrate	0.05	2	2	1	1	2	2	2	2	14	0.005	0.25
	0.005	2	2	1	2	2	2	2	2	15		
	0.0005	2	2	1	1	2	2	1	2	13		
	0.00005	1	2	1	2	2	2	1	1	12		
	0.000005	1	1	1	2	1	1	1	1	9		

* (1) slight growth; (2) good growth; (½) few colonies.

cent potassium citrate or sodium citrate to keep the phosphates in the agar in solution (Bushnell and Hudson, 1927), and various dilutions and combinations of the other five salts were added before sterilization. Inoculation of these media were made from saline emulsions of twenty-four hour cultures of five test strains of *Pasteurella boviseptica*. The amount of growth was compared after twenty-four and forty-eight hours incubation.

Glucose was added to some of the agar solutions and it was found that the consistency of the agar was altered by this carbohydrate. Fifteen per cent agar containing potassium citrate, potassium bicarbonate, and ammonium dihydrogen phosphate became too soft for use when 0.1 per cent glucose was added. To obtain a hard enough agar 2.5 per cent agar solutions had to be used. If, however, the amount of glucose did not exceed 0.05 per cent, a satisfactory agar of 1.5 per cent strength could be made. Glycerol increased the toughness of this agar, and 0.5 per cent glycerol added to the medium insures a satisfactory tough surface. As a result of this study a very satisfactory mineral mixture agar was developed.

MINERAL MIXTURE AGAR

The method of preparing this medium is as follows: Grind 1 pound of beef beart free from fat, or lean meat, through a meat grinder, add 500 to 1000 cc. water, and set in ice box for fifteen hours. Steam for one hour, cool until the fat hardens and decant. This broth should be used at once or sterilized in 2000 cc. flasks for future use. When ready to be used, this meat decoction should be filtered through cotton till clear and to it should be added the following:

Peptone	20	grams
Agar (Difco Bacto purified)	15 to 20	grams
Potassium citrate	2	grams
Potassium bicarbonate	0.5	gram
Ammonium dihydrogen phosphate	0.6	gram
Ferric ammonium citrate	2.5	gram
Glucose not more than	0.5	gram
Distilled water to make	1000	cc.

Steam for one hour to dissolve the agar, adjust to pH 7.2. Usually not more than 20 cc. of normal sodium hydroxide are required. Do not filter. Tube and sterilize at 10 pounds pressure for forty-five minutes.

Difco proteose peptone slightly increases the longevity of Pasteurella organisms grown on this medium.

This mineral mixture agar made without potassium citrate was found to contain a slight precipitate after sterilization, but was slightly superior to the complete mixture for the cultivation of old dried up cultures of *Pasteurella boviseptica*. The dried growth should be first washed off with sterile saline solution before transfers are made.

The virulence of three strains of *Pasteurella boviseptica* was increased by the use of this agar medium. Two strains, nos. 2 and 5, were obtained from another laboratory. Both strains were non-pathogenic for either guinea pigs or rabbits in doses up to 2 cc. of a 0.5 cm. emulsion, the density being determined by the Gates (1920) disappearing loop method. Culture 8, a buffalo strain, lost its virulence during routine cultivation; a few transfers on this medium rendered them pathogenic in doses of 0.05 cc.

The viability of cultures of *Pasteurella boviseptica* grown on this medium were at least equal to the viability of the same cultures grown on hemolyzed blood agar. All strains remained viable for over four weeks and a number of strains grown for some time on this medium remained viable for six weeks.

Eight out of twelve cultures kept at room temperature remained viable for eight weeks, while only four of these cultures remained viable for seven weeks when kept in the ice box.

Salmonella pullorum and *Brucella abortus* grow very profusely on this agar medium.

In the course of the study of the action of salts on anaerobic organisms it was found that certain salts stimulated fermentation to a very great extent. Ammonium dihydrogen phosphate, ferric ammonium citrate, ferric ammonium sulfate, ferrous sulfate, magnesium sulfate, sodium phosphate, cystin, and tyrosin were found to be the only salts among the 68 tested that regularly stimulated the fermentation of sucrose by *Cl. chauvei* in water

containing 0.5 per cent peptone. A very efficient sugar free medium for use in the fermentation of carbohydrates by anaerobes was developed. This medium contained ferric ammonium citrate 0.03 per cent, potassium bicarbonate 0.05 per cent, disodium hydrogen phosphate 0.17 per cent, and ammonium dihydrogen phosphate 0.06 per cent.

The mineral mixture used for the production of mineral mixture agar, and the anaerobic sugar free medium were used as a basis for the development of a sugar free medium for Pasteurella organisms. These two media and twelve combinations of the salts were compared. It was found that a medium containing:

Peptone	2 grams
Potassium bicarbonate	0.5 gram
Ammonium dihydrogen phosphate	0.6 gram
Disodium hydrogen phosphate	1.7 grams
Ferric ammonium citrate	2.5 grams
Distilled water	1000 cc.

proved very satisfactory for the differentiation of Pasteurella organisms from other gram negative organisms isolated from cases of shipping fever. A number of gas forming organisms produced much more gas in this mineral mixture medium than in ordinary sugar free broth.

SUMMARY

1. The growth of *Pasteurella boviseptica* on agar containing different concentrations of six mineral salts is discussed and the optimum concentration of each salt is determined.

2. A mineral mixture agar is described which increases the growth, viability, and pathogenicity of *Pasteurella boviseptica*.

3. The viability of cultures of *Pasteurella boviseptica* is prolonged by storage at room temperature instead of at an ice box temperature of 50°C. or less.

4. A sugar free mineral mixture medium for the differentiation of *Pasteurella boviseptica* from related organisms is described.

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THE BACTERIOPHAGY OF A SPORE-FORMING ORGANISM

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Since the discovery of the first bacteriophage by d'Herelle in 1916 there have been isolated many races virulent for a wide variety of bacteria. It is an interesting fact, however, that of the organisms for which specific races of bacteriophage have been found almost all are non-sporulating. d'Herelle (1926) himself mentions the discovery of a bacteriophage for *B. subtilis*, but reports no study of it. Koser (1926–27) isolated one for a thermophilic spore-forming rod, but was interested in it chiefly from the temperature standpoint. More recently Adant (1928) has described a bacteriophage active against several strains of *B. subtilis*.

In view of this scarcity of information about bacteriophagy among spore-forming organisms, and since a study of this problem might conceivably throw new light on the lytic process and, perhaps, upon sporulation as well, it seemed of interest to attempt some investigations in this field. This paper is concerned with a description of two lytic principles active against *B. megatherium*, a common aerobic spore-former.

B. megatherium was selected for study because, in contrast to most similar organisms, it grows with diffuse turbidity in broth and develops on agar with a relatively homogeneous growth which is easily emulsified. Moreover, it is one of the largest of the bacteria, and as such lends itself especially readily to microscopic observation, even with so low a magnification as that afforded by the 16 mm. objective. A stock laboratory strain (no. 1) was used in the isolation of the bacteriophages and in all routine

work, but six other strains were found to be susceptible. These included another laboratory strain (no. 2), a culture labeled N.Y.U., the American Type Culture Collection strains nos. 72 and 270, as well as two strains (nos. 20 and 20A) obtained from Dr. W. W. Ford. All produced, on agar and potato, the creamy white or yellowish growth, later changing to brown, which is characteristic of the species. The Type Culture Collection strains nos. 71 and 943, listed as *B. megatherium*, were not sensitive to the bacteriophage, and on agar did not develop as this organism usually does.

In all routine work young organisms from an eighteen- to twenty-four-hour agar slant were used. The culture media regularly employed consisted of alkaline extract broth, pH 7.8, and nutrient extract agar of pH 7.0. Filtrations were made through L5 Chamberland candles, and all cultures and culture-phage mixtures were incubated at 30°C.

ISOLATION OF LYTIC PRINCIPLES

Sewage was used as a possible source of lytic principles. This was first filtered through paper to remove the coarser particles, and then 90 cc. of this filtrate were added to 10 cc. of broth made up in ten-fold strength. The reaction of the mixture was tested and, when necessary, sufficient N/10 sodium hydroxide was added to bring the pH to about 7.6, after which the material was passed through a candle. Ten cubic centimeter amounts of this clear filtrate broth were inoculated with enough young megatherium organisms to produce distinct turbidity, and the culture was incubated. Control broth cultures of the same turbidity were prepared each time, in order that any differences in growth might be observed. After twenty-four hours the sewage-culture mixture was filtered, and the filtrate inoculated and incubated. This process was repeated daily, with the addition of sterile broth when needed to replace the loss in filtration. The first three passages showed no difference in turbidity from that of the controls, nor did 0.1 cc. amounts of the filtrate spread over heavily seeded agar plates give visible plaque formation. The fourth transfer, however, seemed slightly less cloudy than the

control, and on plates the filtrate from this transfer showed many areas somewhat more translucent than the body of the culture. Successive passages resulted in the rapid development of lytic power, and the tenth filtrate produced complete clearing of broth suspensions in a dilution of 10^{-6}, as well as,—in proper dilution,—clearly defined plaques on agar.

By the use of the same method, there was obtained from another sample of sewage, several months later, a second lytic filtrate.

It may be mentioned that twenty successive passages in which *B. megatherium* was grown in its own broth culture filtrates failed to show the development of any lytic principle whatsoever. Most of the criticisms directed against experiments which have apparently shown the "spontaneous generation" of bacteriophage have stressed the fact that the original culture perhaps contained a minimum amount of the latent principle. By using spores heated at 90°C. for fifteen minutes, as was done in this case, any bacteriophage present was inactivated, and this criticism could be avoided.

The two lytic filtrates seemed to be essentially alike. They were active in the same dilutions, produced similar plaques on agar, and were heat labile. Fifteen successive passages of filtrates, which had been heated at 80°C. for ten minutes, failed to show any regeneration of lytic power, and other tests showed that a temperature of 75°C. was sufficient to inactivate them. This sensitivity to heat is wholly in accord with what is known about the behavior of other bacteriophages, and, together with other points of similarity, is strongly suggestive that these lytic principles are of the same nature as those for non-spore-forming bacteria.

No secondary growth has as yet been observed in filtrates prepared in the usual way and kept at room temperature for three months. In broth cultures which have undergone apparently complete lysis, however, secondary growth may occur, sometimes within several days, and sometimes not until weeks have passed. This growth is usually scanty in amount and flocculent in character, made up of what appear to be typical organisms.

The fact that the bacteriophage is destroyed by heat makes it possible at any time to eliminate it from a culture, provided spores are present, as is usually the case. Abundant growth results when a clear, lysed culture is exposed to a temperature of 80°C. for ten minutes.

Spores do not seem to be actively attacked by the bacteriophage but will not germinate in its presence. Microscopic observation of spore-phage mixtures on agar shows no change in twenty-four

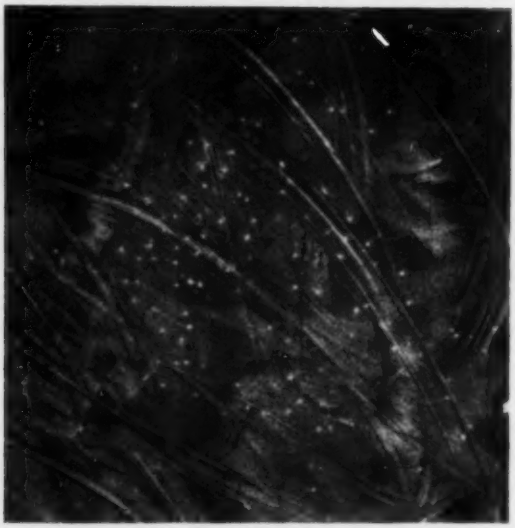

FIG. 1. β PLAQUES, 2 DAYS, SHOWING PLAQUES WITH ONLY A SUGGESTION OF ZONE FORMATION. 3 ×

hours, and inoculations of spores from such mixture into broth fail to develop on account of the bacteriostatic action of the bacteriophage carried over with them. Spores seeded on the surface of an agar plate which had previously been flooded with bacteriophage and dried, failed to grow, but when inoculations were made from such a plate into broth, and then heated, abundant growth occurred. If duplicate bacteriophage titrations are prepared, containing as indicator in one case a visible number of

young megatherium organisms, and in the other so few spores that no turbidity is produced, and then incubated, they will present the same appearance. In the first case the turbidity will have disappeared in the first six tubes (10^{-6}), for instance, although persisting in the last tubes, while in the second case the first six tubes will remain clear, as the bacteriophage has inhibited the development of the spores, and growth will have occurred in the others. This aspect of the problem will receive further study.

FIG. 2. α PLAQUES, 1 DAY, SHOWING DEFINITE DIFFUSION ZONES. 3 ×

It was stated above that the plaques formed by both lytic filtrates are similar. This is true, but in each case these plaques can be divided into two main types which will be referred to for convenience as the α and β forms. The clear plaque proper with both types is 0.5 mm. or less in diameter, and does not increase in size upon prolonged incubation. The α principle, however, is characterized by the ability to diffuse from the plaque through the surrounding culture with the production of a translucent zone, which, upon incubation for a week or two may attain a

width of 10 mm. or more. During the first few hours of incubation the zone formed may be so clear as to suggest that it is identical with the plaque, but microscopic observation reveals the presence of numerous organisms, increasing in number as the distance from the true plaque increases. The outer parts of this diffusion zone, at any stage of its development, are composed of masses of spores with occasional vegetative rods, and shadow forms.

The β principle, as a rule, does not show the presence of a diffusion zone, and microscopic observation of a β plaque reveals a more sharply defined boundary than is seen with the α plaque.

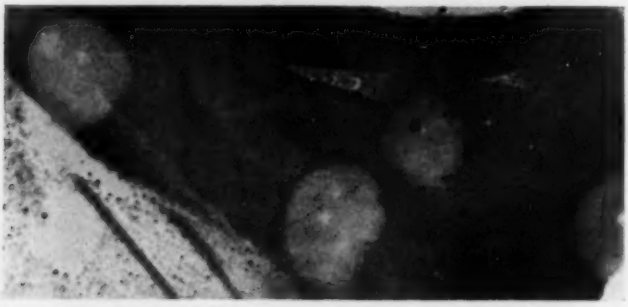

FIG. 3. α PLAQUES, 5 DAYS, SHOWING EXTENSIVE DIFFUSION OF BACTERIOPHAGE. 3 ×

Both principles can be recovered from their respective plaques or from any portion of the zone, when this is present, and, as far as can be told, both principles continue to breed true.

It should be borne in mind that all of these descriptions refer to observations made on only one strain of *B. megatherium* growing on the usual nutrient extract agar. Numerous observations have indicated that the strain of culture used and especially the amount of nutritive material available have pronounced effects upon the process of bacteriophagy. Details regarding the intimate behavior of the two types of bacteriophage under varying conditions and with diverse strains of *B. megatherium* will be presented in a later paper.

MICROSCOPIC OBSERVATION OF LYSIS

As has been mentioned, the large size of *B. megatherium* facilitates microscopic study and, hence, permits the lytic process to be followed more readily than is possible with the smaller bacteria which have heretofore been used.

In preliminary experiments the ordinary hanging drop was tried, but the active motility of the organism rendered this method unsatisfactory. As a substitute the following technic was employed. A drop of melted agar is spread over the surface of a sterile cover-slip and allowed to harden. A small amount of broth containing both megatherium cells and bacteriophage is next placed on the agar and dried. The cover-slip is then inverted on a concave slide and sealed with vaseline, after which the slide is placed on the warm stage under the 4 mm. objective of a microscope.

When a suitable field, containing perhaps a dozen or more cells, is found, it is charted on paper for easy reference and then observed at intervals until growth is apparent. This occurs, as a rule, in from one to three hours, and at about the same time lysis may be seen. Generally lysis is not evident before actual cell division has been noticed, although at times a single organism may vanish before there has been any apparent growth. The dissolution of the bacteria is not instantaneous. Instead, the organism seems to become more and more transparent, it cannot be focused sharply, and, finally, in from five to ten seconds after the first change has been noticed, it fades away entirely. There is no apparent gradual decrease in size such as exists when a substance dissolves, and there is no visible residue, although occasionally there may be seen, occupying the site of the cell, a faint shadow which usually vanishes within a few minutes. It is worthy of note that in numerous observations of the lysis of *B. megatherium* there has never been seen any swelling or rupture such as have been reported with other bacteria.

The following observations, typical of many others, may be briefly described. A single organism was watched while it grew into a chain of three. An end cell of this chain underwent lysis,

but the two remaining cells continued to divide, growing over the spot previously occupied by the cell which had vanished. When this chain had grown to comprise five cells it broke apart and at length another end cell disappeared, twenty-five minutes after the first had gone. The end cells need not be the first affected however. A chain of four or more may undergo lysis as a whole, or the middle one of a series may succumb. Sometimes one cell of a pair will disappear, while the other will resist the lytic action for many minutes. Frequently a typical raft of many cells will develop, only to undergo lysis eventually, both from within and without.

A preparation in which bacteriophage was acting on organisms from a four-day agar culture contained many cells which failed to grow. Some of these seemed to be susceptible to the lytic agent, but required periods of several minutes to an hour between the initial change and final dissolution. Heat killed organisms were observed for twenty-four hours without the appearance of any lysis.

All of these facts are quite in accord with the usual statements that the most actively growing organisms are the most susceptible to the bacteriophage, while old cells are less so, and dead cells, except perhaps in the presence of living ones, are not affected at all.

SUMMARY

The isolation of two bacteriophages specific for *B. megatherium*, a common aerobic spore-forming organism, is described. Various strains of this organism, seven in number, obtained from different sources, were susceptible to the lytic action.

Each bacteriophage contained two principles, one of which was characterized by the ability to diffuse from the initial plaque and cause changes in the surrounding culture.

Spores seem to be resistant to the action of the bacteriophage, but are inhibited from developing by its presence.

The lytic principles were destroyed by exposure to a temperature of 75°C. for ten minutes, and could in this way be eliminated from cultures containing organisms in the spore stage.

The lytic process as seen under the microscope is described. *B. megatherium* does not, apparently, swell or burst in lysis but rather fades from view.

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PROTOBACTERIAL FORMS OF B. DIPHTHERIAE

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Among the more important of the recent developments within the field of bacteriology must certainly be included the recognition of the fact that under certain circumstances, at least, bacteria pass through, or yield, forms which are filter-passing,—the so-called protobacteria. That such forms exist seems to have been established beyond question, despite the fact that the factors controlling filtrability or non-filtrability are still, in many respects, obscure. It may readily be supposed that further studies of the physical conditions determining filtrability and, perhaps, also of the biological states permitting or facilitating filtration will serve to define more precisely the nature of the beings involved, and to explain the relationships between complexity of organization and antigenic and biochemical attributes.

Just as it has been impossible to consider the bacterium in its visible form without taking cognizance of the environment within which it exists and upon which it subsists and reacts, so also will it be impossible to regard the protobacterial forms as unassociated with their surroundings; for, although they have thus far been regarded as of significance chiefly from the point of view of ontogeny, it is certain that from the standpoint of phenomena of infection and resistance they must play parts of fundamental importance. It is only necessary to recall the work of Friedberger (1927) Bisceglie, (1924–5) and of Fejgin (1927) upon the kryptantigenic viruses to gain some impression of the possible importance of such forms in reaching valid conclusions concerning problems of epidemiology, immunology, and preventive medicine.

In view of the accumulated reports upon the subject, it is so

longer necessary to adduce proofs that bacteria may exist in, or yield, the protobacterial stage. Furthermore, the diversity of the bacterial species for which such forms have been described clearly shows that the monomorphic concept is not one to which there are certain isolated exceptions, but rather that, if exceptions must be taken, the instances where protobacteria have not been demonstrated must be so regarded, and that polymorphism must be accepted as the general rule. It surely would be difficult to find organisms more divergent, from all recognized methods of differentiation, than *B. coli* and *B. pestis*, *B. typhosus* and *B. fusiformis*, the paratyphoid bacilli and the staphylococcus, *B. dysenteriae* and *B. diphtheriae*, *B. proteus* and *B. tuberculosis*, or *V. cholerae* and the streptococcus, and yet for all of these, filtrable forms have been disclosed. It does not seem unreasonable to infer that with the application of proper methods this list can be extended to embrace all species; or, indeed, it may very well be that such study will but serve to show that many of the forms now recognized as species are derivatives, through the filter-passing form, of other species.

Such questions remain to be answered, as does that bearing on the nature of the incitants which may lead to the appearance of protobacteria. Whether they originate solely through the operation of some external force or whether they represent some obligatory or optional stage in cyclogeny is a matter of the greatest significance. Manifestly, with the fragmentary data now available it is premature to attempt broad generalizations or to formulate a theory sufficiently comprehensive to explain the phenomena observed or to suggest the significance of the facts now known.

The period of observation and of the mere recording of facts can well be extended, leaving speculation, attractive as it may be, until further disclosures have measurably filled the obvious gaps in our knowledge. Thus it is that the data presented here comprise the results of studies made upon the presence of protobacterial forms of *B. diphtheriae*, no attempt being made to explain their significance.

The initial purpose of the study was to investigate the possible

TABLE 1

STRAIN DESIGNATION	SOURCE	MORPHOLOGICAL TYPES (INITIAL CULTURES)	MORPHOLOGICAL TYPES (CULTURES FROM RESIDUES)
Co...	New Haven Hospital	a, a′, a^2	c^2, coccoid forms
Ha...	New Haven Hospital	a, a^2, c, d	c^2, d^2
Tr...	New Haven Hospital	a, a′, a^2, c^2, d^2	d^2
En...	New Haven Hospital	c, c^2	c^2, d^2
Pa...	New Haven Hospital	c, c^2	No growth
Hay...	New Haven Hospital	a, a′, a^2, c, c′, d, d′, d^2	a^2, c^2, d^2
Hay-1...*		a, a′	d, d^2
Hay-2...*		c, c′, d, d′	a^2, c^2
Park-Williams No. 8			d^2
Park-Williams No. 8 + resistant staphylococci			d^2
Park-Williams No. 8 + susceptible staphylococci			d^2
No. 721	Connecticut State Board of Health	c, c′, d, d′, d^2	c′ (few), d^2
No. 955	Connecticut State Board of Health	d, d^2	d^2 (atypical)
No. 5	Connecticut State Board of Health	c, c′, d, d′	d′, d^2
No. 9	Connecticut State Board of Health	d, d^2	d^2
No. 959	Connecticut State Board of Health	c^2, d^2	No growth
No. 720	Connecticut State Board of Health	a^2, c^2, d, d^2	c^2, d^2
No. 723	Connecticut State Board of Health	c, c^2, d	d′, d^2
No. 721–10	Connecticut State Board of Health	c, d	c, c′, c^2

* Cultures Hay-1 and Hay-2 were derived from the original Hay culture as obtained from a clinical case of diphtheria. As is indicated above, this culture showed a wide variety of morphological types. Platings from a single colony yielded colonies of two types: 1, a large colony comprised of large, heavily staining club-shaped bacilli exhibiting marked pleomorphism; 2, a minute colony in which small beaded bacilli predominated with but an occasional swollen cell. The original culture, which was made from a single colony, and the two derivative cultures were treated as three strains.

relation between diphtheria toxin, and toxin production, and the capacity of diphtheria strains to yield filtrable forms, a problem obviously suggested by the work of Hauduroy (1927) who succeeded in demonstrating filter-passing forms in a number of samples of diphtheria toxin. These organisms, derived from his Chamberland filtrates, possessed the morphological and biochemical characteristics of *B. diphtheriae*, but were lacking in pathogenic properties for the guinea pig, an alteration in character which seems to be shared by the majority of protobacterial forms.

In order to initiate the study, pure cultures of 17 strains of *B. diphtheriae* were obtained; including 8 isolated from clinical material in the New Haven Hospital, 8 strains obtained from the Connecticut State Board of Health Laboratories, and the standard Park-Williams No. 8 which is used in toxin production. That the capacity to yield filtrable forms might be in some way related to type was, of course, obvious; hence the types of organism present in the original cultures were made a matter of record, as appears in table 1.

For obtaining toxin production the several strains were grown, with repeated daily transfers, in a nutrient broth in order to habituate the organisms to the medium somewhat prior to the inoculation of the larger quantities requisite to obtaining the desired amount of toxin. For the latter, large erlenmeyer flasks were employed, thus presenting a large surface for aerobic growth. With the cultures which readily developed a pellicle a portion of this was floated upon the surface; with the strains where pellicle formation was restricted, sterile, thin cork discs were coated with agar, and these, after inoculation with the cultures in question, were floated upon the surface of the broth. In all instances growth, with pellicle formation, took place.

In all save two instances the flasks were inoculated with known pure cultures of *B. diphtheriae;* the two exceptions being flasks planted with known mixed cultures, one containing the diphtheria bacillus (P-W No. 8) in association with a bacteriophage-susceptible strain of the staphylococcus, the other with the same strain of diphtheria organism in combination with a bacteriophage-resistant derivative of the same strain of staphylococcus. The

particular purpose in this procedure was, of course, quite outside of the primary purpose of the study, namely, to investigate the question of bacterial associations as related to the development of toxins and their behavior in the skin test. These problems have no bearing upon the matter under discussion, but are mentioned simply by way of explaining the reason for employing such mixed cultures and, as will be seen in the following discussion, it is possible that such associations may be of some significance in the origin of protobacterial forms. Indeed, it may very well be that under normal circumstances, biologic antagonisms or symbioses may be incitants of cyclic changes in the beings involved.

It may be well, before presenting the results of the studies with these diphtheria cultures, to state that the primary object—a study of the toxins produced—has not been reached, consequently no data will here be presented bearing upon the possible relation between toxin and protobacterial forms.

The broth inoculations with the diphtheria strains were made in the month of April, 1929. Because of circumstances it was impossible to prepare the necessary filtrates for testing, both for toxin and for protobacterial forms, as had been planned. Indeed, it was not until October, six months after inoculation, that they received attention. During the intervening period the flasks had remained at room temperature, carefully closed, but not sealed in a way to prevent evaporation, and when subjected to further examination the contents had in most instances been reduced through loss of water to a thick syrup or gum-like residue.

To permit further study and filtration this concentrated material was redissolved in sterile distilled water. Of the resuspended material smears and cultures, as well as filtrates, were made. Neither the smears nor the cultures showed evidences of contamination and, aside from the two instances which yielded no growth, the cultures derived corresponded in type fairly well, after several transplants, with the types present in the initial cultures inoculated. The types observed are recorded in table 1 in parallel with the initial types; and if such observations have significance it would seem that the tendency

for organisms of the d^2 type to persist or to appear in the cultures is the only salient feature, aside from the fact that there is an almost complete disappearance of the beaded and barred forms.

In view of the known transformations from one type of morphology into another, this change from the beaded and barred types into the solid-staining forms is not surprising, particularly since organisms of this type are considered as the more saprophytic forms and thus are presumably more competent to withstand adverse environmental conditions. However this may be, it is significant that throughout a period of six months the organisms remained viable in a medium constantly becoming more and more concentrated, and consequently exerting a greater and greater physical force upon the organisms present within it. It would seem that a degree of adaptation must have been brought about; and it is, of course, possible that such transformations in physical state must needs have their counterpart in changes in morphological structure. In contrast to this rather surprising viability of these cultures under such adverse conditions it is of interest to note that pure cultures of the initial strains which had been in sealed tubes of Loeffler's medium in the ice-box throughout this same period failed to yield growth when subcultured.

That such changes in morphology as are evidenced by the data presented in table 2 are not necessarily permanent is shown by the fact that with certain of the cultures derived from the medium residues a regression to the original type took place when these were subjected to further cultivation under the ordinary conditions of bacterial growth. Thus, with certain of the strains which have been repeatedly transplanted the solid-staining character has been lost, and after a period of three months they present the Wesbrook morphological types indicated below. In the table are listed the initial types present, the types developing from the concentrated medium upon their first cultivation, and the types ultimately to appear.

With a few of the recovered cultures virulence determinations were made, showing that this attribute seems to bear little if any relation to any other discovered factor; thus, strains Co...,

TABLE 2

STRAIN DESIGNATION	INITIAL TYPES	TRANSITIONAL TYPES	ULTIMATE TYPES
Co...	a, a′, a²	c², coccoid forms	a′, a²
Ha...	a, a², c, d	c², d²	d
P-W. No. 8		d²	d²
No. 721	c, c′, d, d′, d²	c′ (few), d²	a′, c′
No. 955	d, d²	d² (atypical)	d′
No. 5	c, c′, d, d′	d′, d²	c′, d′
No. 9	d, d²	d²	a²
No. 720	a², c², d, d²	c², d²	a′, c′, c², d′
No. 723	c, c², d	d′, d²	a′, c′
No. 721–10	c, d	c, c′, c²	a

TABLE 3

STRAIN DESIGNATION	CULTURAL TYPES	VIRULENCE (CULTURE)	TOXICITY (FILTRATE)	EVIDENCE OF SECONDARY GROWTH
Co...	c², coccoid forms	0	0	+
Ha...	c², d²	0	0	++
Tr...	d²		0	±
En...	c², d²		0	0
Pa...	No growth		0	+
Hay...	a², c², d²		0	0
Hay-1...	d, d²		+	++
Hay-2...	a², c²		0	++
P-W No. 8	d²	0	0	++++
P-W No. 8 + R St.	d²		0	+
P-W No. 8 + S St.	d²		0	0
No. 721	c′ (few), d²	+	+	0
No. 955	d²	0	0	+
No. 5	d′, d²		+	+
No. 9	d²	0	0	±
No. 959	No growth		0	0
No. 720	c², d²	+	+	++++
No. 723	d′, d²	+	0	±
No. 721–10	c, c′, c²	0	+	0

Ha..., P-W No. 8, No. 955, No. 9, and No. 721–10 proved to be avirulent, while strains No. 721, No. 720, and No. 723 were virulent.

As stated above, after redissolving the medium residues and

making cultures from them, these residues were subjected to filtration. In all instances filtration was carried out with the Martin arrangement of apparatus, Chamberland L_5 candles being employed. Inasmuch as these candles had been used before, as they have since, in other filtration work without giving evidence of being in any way defective, there is no reason to suppose that the results obtained in this series are due to faulty filters or technic.

The filtrates were all prepared between November 4 and November 8, and cultures made in broth and in deep agar immediately after the filtration failed in every instance to yield growth. Tests for the toxicity of the filtrates, made by the intracutaneous method in guinea pigs, clearly showed that certain of them were definitely toxic, others were not (table 3).

Following the making of bacteriological controls, the filtrates were allowed to stand for periods of between two and three weeks, with frequent observation in order to detect the appearance of the faint clouding or of the fine sedimented precipitate characteristic of the development of secondary cultures in filtrates prepared in bacteriophagy experiments. In the majority of filtrates this evidence of growth, although stained smears showed nothing, appeared between November 19 and 30. In table 3 the presence of such a sediment, as well as the relative volume, is indicated, and for correlative purposes are given the morphological types present in the residue cultures, the virulence of these cultures, and the results of the intracutaneous toxicity tests.

It is thus apparent that the development of turbidity in filtrates bears no direct relation to the Wesbrook type present in the cultures yielding such filtrates, or to cultural virulence or filtrate toxicity. Apparently these several factors vary independently.

Following the technic of Hauduroy (1927) these filtrates were spread upon plates containing beef infusion agar of the following composition: 1 per cent peptone, 0.08 per cent Na_2HPO_4, and 1 per cent lactose, with phenol red as indicator. The medium contained 1.5 per cent agar. In the inoculation about 0.2 cc. of the filtrate was used, it being spread evenly over a small area of the medium. The plates were incubated at 38°C., and follow-

ing this the area so inoculated was carefully washed off, whether visible change had taken place or not, with a small quantity of pneumococcus broth, and this material was again spread upon a fresh plate. This procedure was repeated at twenty-four to forty-eight-hour intervals, as indicated in table 4. In such serial inoculations the first change to take place in the medium, indicative of growth of any type, was the occurrence of a slight dulling of the otherwise shiny surface. With transmitted light this change was not apparent, but could be detected only by so holding the plate that the light struck it obliquely. In the earlier transfers showing such dulled areas stained smears prepared from the washings failed to show any organized material suggestive of bacterial growth, but with later transfers, as colonies developed, even though they were microscopic in size, smears revealed forms which were, unquestionably, bacteria. These morphological changes will be discussed later.

The number of transfers necessary to yield the dulled surface was variable and seemed to bear no definite relation to the amount of sedimented precipitate present in the original filtrate used as inoculum. Table 4 states the dates of the several spreadings of each of the filtrates, together with appearance of the areas inoculated; + indicating but a very slight dulling of the surface, ++++ meaning that the dulling was pronounced, and "colonies" meaning the appearance of microscopic colonies which, when stained, showed definitely formed microörganisms.

Examination of the table shows that of the nineteen cultures carried through the serial spreadings, colony development took place more or less promptly with eleven. In two instances they appeared on the fifth transfer, in two on the sixth, in two on the seventh, in one on the ninth, in one on the tenth, in one on the eleventh, in one on the twelfth, and in one on the thirteenth. It is probable that colony formation would have been secured in other instances, perhaps, indeed, in all, had it been possible to continue the subculturing. This was, however, impossible, for the study and further cultivation of the positive cultures obtained involved so much technical work that it seemed wiser to make sure of those already recovered, rather than risk losing them through trying to gain growth from all filtrates.

These results do not indicate in any way whether the readiness with which colony formation is obtained is referable to strains which can be cultivated from the protobacterial form with particular ease or whether quantitative relations are significant here, as they are in the growth of the visible forms of bacteria where variations are made in the volume of the inoculum. It is also of significance that in some cases where the plates showing no dulling of the surface were allowed to stand, dulling and colony formation later appeared. This suggests that, had the intervals between transfers been greater, that is, had the plates

TABLE 4

STRAIN DESIGNATION	FIRST SPREADING		SECOND SPREADING		THIRD SPREADING		FOURTH SPREADING		FIFTH SPREADING		SIXTH SPREADING		SEVENTH SPREADING		EIGHT SPREAD
Co...	11.19	0	11.21	0	11.25	+	11.26	0	11.27	0	11.29	0	12.2	∞	12.3
Ha...	11.19	+	11.20	0	11.25	0	11.26	0	11.27	0	11.29	0	12.2	0	12.3
Tr...	11.20	0	12.2	0	12.3	∞	12.4	0	12.7	0	12.9	0	12.10	+	12.14
En...	11.25	+	11.27	∞	11.29	0	12.4	0	12.7	∞	12.11	++++	12.14	0	12.17
Pa...	11.21	0	11.25	++	11.26	++	11.29	++++	12.2	colonies					
Hay...	11.19	0	11.21	0	11.25	0	11.26	0	11.27	∞	11.29	∞	12.2	0	12.3
Hay-1...	11.19	+	11.20	0	11.25	0	11.26	0	11.27	0	11.29	+	12.2	colonies	
Hay-2...	11.19	+	11.21	0	11.25	++++	11.26	++++	11.27	++++	11.29	colonies			
P-W No. 8	11.19	0	11.21	0	11.25	0	11.26	++	11.27	+++	11.29	colonies			
P-W No. 8 + R St.	11.21	0	11.25	++	11.26	++	11.27	++	11.29	++	12.2	0	12.3	0	12.4
P-W No. 8 + S St.	11.19	∞	11.21	0	11.25	0	11.26	+	11.27	colonies					
No. 721	11.19	0	11.25	∞	11.26	++	11.27	∞	11.27	0	11.29	0	12.2	0	12.4
No. 955	11.19	0	11.21	0	11.25	++	11.26	+++	11.27	+	11.29	++	12.2	colonies	
No. 5	11.19	0	11.21	0	11.25	++	11.26	++	11.27	+	11.29	0	12.2	0	12.3
No. 9	11.19	0	11.21	0	11.25	++	11.26	++	11.27	∞	11.29	0	12.2	∞	12.3
No. 959	11.19	0	11.21	0	11.25	0	11.26	0	11.29	0	12.2	0	12.4	0	12.7
No. 720	11.19	∞	11.21	∞	11.25	0	11.26	0	11.27	0	11.29	0	12.2	0	12.3
No. 723	11.21	0	11.25	++	11.26	++	11.27	0	11.29	0	12.2	0	12.3	0	12.4
No. 721-10	11.19	0	11.21	0	11.25	0	11.26	0	11.27	0	11.29	0	12.2	∞	12.3

been held longer before washing, colony appearance might possibly have been encountered in some of the earlier serial transfers.

A correlation of the positive cultures with the other characteristics of the strains shows that the tendency to yield protobacterial forms such as can be cultivated agrees most closely with the tendency of the filtrates to develop a sedimented precipitate upon standing. Even here the correlation is not complete, but agreement between these two characters is far more consistent than between colony formation and filtrate toxicity, derived

morphologic type or original morphologic form. But three of the five filtrates which showed toxicity yielded colonies, and in these growth was not particularly prompt.

The behavior of the Hay... cultures and of the Park-Williams No. 8 mixtures is of interest, but possibly of no great significance. The original Hay... culture showed no filtrate precipitate and failed to dull the surface of the medium or yield colonies, while of the two derivative strains, Hay-1 and Hay-2, both gave a moderate amount of filtrate precipitate and both yielded colonies, Hay-1 (the large colony, club-shaped bacillus strain) on the

TABLE 4

ENTH ADING	EIGHTH SPREADING		NINTH SPREADING		TENTH SPREADING		ELEVENTH SPREADING		TWELFTH SPREADING		THIRTEENTH SPREADING		FOURTEENTH SPREADING		FIFTEENTH SPREADING	
∞	12.3	0	12.7	0	12.9	0	12.11	0	12.14	0	12.17	0	12.19	0		
0	12.3	0	12.4	++	12.7	0	12.9	++	12.10	0	12.14	0	12.17	0		
+	12.14	0	12.17	colonies												
0	12.17	0	12.26	0	1.6	colonies										
0 colonies	12.3	∞	12.4	0	12.7	0	12.9	0	12.10	0	12.14	0	12.17	0		
0	12.4	0	12.7	0	12.9	0	12.11	0	12.14	0						
0 colonies	12.4	++	12.9	0	12.9	0	12.11	0								
0	12.3	0	12.4	++	12.7	+++	12.9	+++	12.11	colonies						
∞	12.3	0	12.4	0	12.7	0	12.9	0								
0	12.7	0	12.9	0	12.11	0	12.13	0	12.14	0	12.17	0	12.19	0	12.26	0
0	12.3	0	12.4	∞	12.7	0	12.9	0	12.11	∞	12.17	colonies				
0	12.4	0	12.7	0	12.9	0	12.14	colonies	(colonies later developed on the 12.9 plate)							
∞	12.3	∞	12.4	++	12.7	0	12.9	++	12.17	0	12.26	0				

seventh passage, and Hay-2 (the minute colony composed of beaded bacilli) on the sixth spreading. The colonies made their appearance suddenly in the case of Hay-1, whereas with Hay-2 a marked clouding of the surface had been evident from the third passage.

In the case of the Park-Williams strain, a race of diphtheria bacilli well known to be lacking in virulence, but of high toxigenic power, colonies developed on the sixth transfer. The results with this strain grown in association with staphylococci of the bacteriophage-susceptible and bacteriophage-resistant varieties were

clearly different. Such variations in behavior as those associated with these cultures may very well have been quite accidental. The only statement that can be made with any assurance is that with eleven out of nineteen filtrates cultures have been recovered which may very well represent development from protobacterial forms of *B. diphtheriae*. The causes and mechanisms leading to this result are obscure.

Study of the characteristics of the organisms ultimately developing from the filtrates involves a consideration of their morphology, their colony type and pigmentation, and their biochemical activities. Serological studies upon these strains have not yet been completed.

As stated above, the development of colony formation upon the plates is usually preceded by a progressive dulling of the surface of the medium throughout the successive transfers. Smear preparations from such dulled areas fail uniformly to show bodies having a recognizable morphology. The stainable amorphous material may present an occasional Gram-positive granule more deeply stained than the surrounding mass which is Gram-negative, but these granules possess no distinctive characteristics and they may or may not represent the precursors of the organisms later to develop. With approaching colony formation on the plates, the smears first show large, diffuse, fairly round bodies, usually about 4μ in diameter; and somewhat later these tend to give place to an abundance of granules, definitely coccoid in form, and often appearing in pairs, or they may be supplanted by clearly defined, large, clumsy rod-shaped structures. The latter exhibit a marked degree of pleomorphism and extreme variation in size. Swollen club-shaped forms predominate, and, as colony development on the plates progresses, these rods may in turn give way to the development of bacillary forms which morphologically can not be differentiated from certain types of *B. diphtheriae* or from the diphtheroids. They are Gram-positive, and not acid-fast, and when suitably stained they may or may not show metachromatic granules.

In the development of the colonies upon the plates a progressive increase in the size of the colony and luxuriance of the growth becomes apparent with the first few transfers. The initial colony

to appear is microscopic in size, smooth in character, flat, even in contour, transparent, and dull. With subculturing, growth becomes more and more luxuriant and pigmentation of the colony may become an outstanding characteristic, together with the assuming of a markedly mucoid character. These characters resemble closely those so commonly attributed to certain varieties of the diphtheroids, and are not unlike the pigmented strains of diphtheria described by Barratt (1924–5).

The organisms composing the final, derived cultures exhibit a wide degree of morphology, with, in almost all instances, an admixture of coccus-like forms and rod-shaped bacilli. A tabulation of the records made with reference to each of the derived strains shows the following:

Strain Tr... Colonies white and transparent. Organisms granular and coccoid, with solid staining rods. Colonies appeared on ninth passage.

Strain En... Colonies minute, white. Organisms small cocci, irregular in size, diplococci, rods. Colonies appeared on ninth passage.

Strain Pa... Colonies yellow-white, growth scanty. Organisms irregularly-shaped coarse rods, solid-staining, and occasional diplococci. Colonies appeared on fifth passage.

Strain Hay-1... Colonies yellow-orange. Organisms granules, cocci, and cocco-bacilli. Colonies appeared on seventh passage.

Strain Hay-2... Colonies white, with small daughter colonies orange in color superimposed upon the white growth in older cultures. Organisms, granules and cocci, large rods, with swollen ends, containing granules. Colonies appeared on sixth passage.

Park-Williams No. 8... Colonies yellow and mucoid (apparently identical with those recovered from the broth culture after standing). Organisms very small and faintly staining rods in the first passages, later of the typical d^2 type. Colonies appeared on sixth passage.

Park-Williams No. 8 in combination with bacteriophage-resistant staphylococci. Colonies yellow and mucoid. Organisms small rods at first, later larger and of the d^2 type. Colonies appeared on fifth passage.

Strain No. 955... Colonies white or gray; almost transparent. Organisms coccoid, large, irregularly-shaped rods. Colonies appeared on seventh passage.

Strain No. 5... Colonies yellow and mucoid. Organisms cocci and rods with granules. Colonies appeared on twelfth passage.

Strain No. 720... Colonies small, transparent. Organisms heavy club-shaped rods. Colonies appeared on thirteenth passage.

Strain No. 723... Colonies very minute, non-pigmented. This growth appeared on the plate of the eleventh passage after it had been inoculated for two weeks. Subcutures failed to grow. Organisms small wedge-shaped rods and diplococci.

It is difficult to detect any direct connection between the final type of colony and the morphology of the organisms composing it, and the nature of the initial cultures or of the cultures recovered from the residues; nor does there seem to be any relation between the readiness with which growth was obtained and the type of organism present.

None of the cultures recovered from the filtrates possess virulence. This finding is, of course quite in accord with the results of most observers who have regained cultures from protobacterial forms. The serological and antigenic behavior of these derivative cultures, as well as of the stages intermediary between the protobacterial form and the final form, remain to be determined.

It may be unwise to mention the fermentative reactions exhibited by the cultures recovered from the filtrates, for if bacteriologic experience indicates anything it shows that fermentation characters are most unstable; that in dissociative phenomena the derivative strains may possess attributes quite unlike those exhibited by the parent strain. Thus, without placing emphasis upon this point, the activities of nine of the diverse strains recovered from the protobacterial forms upon a few of the more common carbohydrates are here recorded (table 5).

Whatever significance these results may have, it appears that three types of organism were derived; three strains corresponding to *B. diphtheriae* in so far as glucose and dextrin fermentation is concerned: 5 strains corresponding to *B. hoffmanii*, and one

strain, which, because of its ability to ferment lactose, may perhaps belong to the enzymicus group. More important than allocation to groups, however, is the fact that derivatives from the protobacterial forms show different attributes; indicating that such a transformation in state offers further means for variability rather than a mechanism for regaining or re-establishing a fixed type.

It is impossible to consider the development of the diphtheroid forms from filtrates of cultures, which by all ordinary criteria must be classed as diphtheria (it may be remarked, that every culture used in this series was designated as *B. diphtheriae* by some other laboratory before it reached our hands) without

TABLE 5

STRAIN DESIGNATION	GLUCOSE	LACTOSE	SUCROSE	MALTOSE	SALICIN	DEXTRIN
Tr...	+	+	+	+	−	−
En...	−	−	−	−	−	−
Pa...	+	−	−	−	−	+
Hay-1...	−	−	−	−	−	−
Hay-2...	−	−	−	−	−	−
P-W No. 8	−	−	−	−	−	−
No. 955	−	−	−	−	−	−
No. 5	+	−	−	−	−	+
No. 720	+	−	−	−	−	+

recalling the work of Mellon (1917) upon this group, and the more recent work of Walker (1929), and of Ramsin (1926), Hauduroy (1927), and others. The frequency with which organisms of this type appear, whatever may have been the nature of the original culture type under study, suggests that the diphtheroids represent a developmental stage in the cyclogeny of many bacterial species rather than a species *sui generis*. This suggestion has already been made by Koch and Mellon (1930) and the results here reported merely add to the evidence already accumulated.

CONCLUSIONS

From broth cultures of *B. diphtheriae* which have undergone aging, with loss in water through evaporation, filtrates may be

obtained which upon serial transfers yield visible growth. From eleven out of nineteen such cultures positive results have been obtained.

The morphological types of the organisms ultimately to develop include forms which can not be differentiated from bacilli of the diphtheroid group.

In the development of visible forms of bacteria from the protobacterial forms, a more or less constant sequence of morphologic types obtains: a granular stage, followed by the appearance of giant cocci which are replaced by micro- and diplococci, and ultimately by pleomorphic bacillary forms.

The readiness with which such protobacterial forms develop can not, at present, be correlated with any attribute of the organisms subjected to test.

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STUDIES IN BACTERIAL PROTEASES

I. THE RELATION OF PROTEASE PRODUCTION TO THE CULTURE MEDIUM

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The differences in protease producing power observed in the same microörganism under different cultural conditions have led to the conception that the culture medium must supply some essential material for the elaboration of the enzyme, apart from those necessary for the ordinary metabolic processes of the bacterial cell. With one exception emphasis has been laid upon the part played by the nitrogen constituents of the medium, the general conclusion of many workers being that without organic nitrogen growth is possible, but not protease formation. Fermi in his early work on bacterial proteases found only two organisms, *B. prodigiosus* and *B. pyocyaneus*, that produced protease when grown in a synthetic medium with an inorganic source of nitrogen. Drummond (1914) concluded that protease could be derived from nothing less complex than a peptone and hypothesized the formation of a preliminary compound of enzyme and peptone which attacked the protein, setting free peptone which could then activate the enzyme for further protein-splitting. Jordan (1906) found that asparagin made a suitable culture medium for protease production. Diehl (1919) also concluded that simple protein cleavage-products, such as amino-acids, were satisfactory sources of nitrogen for protease formation, but added the very attractive theory of a specificity of proteases for the amino-acid nuclei of proteins. He found for example that a gelatinase but no caseinase was produced in culture upon glycocoll, of which gelatin has a high content, whereas

the reverse held for cultures upon tyrosine which is abundant in casein but lacking in gelatin. The writer has been unable to confirm this specificity of protease in cultures of *B. subtilis* in glycocoll and in alanin. Neither Drummond nor Diehl were able to demonstrate protease in cultures on a synthetic medium containing inorganic nitrogen, in spite of good growth.

Waksman (1918) reported protease production by some of the higher fungi in a nitrate-sucrose medium. Merrill and Clark (1928) have also found that inorganic nitrogen suffices for the production of protease. Their work, however, removes the emphasis from the nitrogen constituents of the medium and places it upon the salt content. They find that *B. proteus-vulgaris* grown in a buffered medium of inorganic nitrogen with glucose, glycerol or a lactate as a source of carbon, will produce only traces of gelatinase unless there is added to the medium a salt of calcium or of magnesium. If both calcium and magnesium are added, the gelatinolytic capacity of the culture increases still more, an increase which is apparently due to an effect independent of any growth stimulating effect of the combined ions.

Indirectly, the need for an active nitrogen metabolism in protease formation is assumed in the considerable discussion that has centered about the effect upon protease production of an alternative source of carbon in the culture medium. From studies of the nitrogen metabolism of various bacteria in conjunction with examinations for the presence of protease, Kendall and his coworkers have concluded that the delayed formation or complete absence of protease which they found in cultures in a protein medium to which had been added a fermentable sugar is to be explained on the basis of a "protein-sparing" action of carbohydrate, comparable to that found in mammalian metabolism. Jones has confirmed the finding that a fermentable carbohydrate delays or inhibits protease formation. Waksman, however, finds that for *Aspergillus niger* this does not hold true, as protease is formed as well in a glucose-peptone medium as in plain peptone. Merrill and Clark also report several instances of "active" protease production by *B. proteus-vulgaris* in a 2 per cent glucose-peptone medium. The studies of Jordan include instances of

increased gelatinase production upon the addition to an asparagin medium of lactose and glycerol.

Raistrick and Clark studied the effect of an alternative source of carbon upon the nitrogen metabolism of *B. coli* and found that the utilization of nitrogen in the presence of a second source of carbon, glycerol, was increased rather than decreased. Similar studies by Rettger and his associates of the proteolytic bacteria have also demonstrated that in suitably buffered peptone media the addition of glucose aids in the utilization of nitrogen. But it is not shown in their papers that a soluble proteolytic enzyme can be demonstrated as readily in a glucose-containing as in a plain peptone medium. Experiments to be discussed below show that the presence of an alternative source of carbon in protein medium, or in a synthetic medium containing the ammonium salt of an organic acid as the source of nitrogen, does not interfere with protease formation.

Growth as the deciding factor in protease production has had but one advocate. Malfitano (1890) studied the proteolytic behavior of cultures of *Aspergillus niger* in relation to the stages of fungus growth, and found a direct relation between the development of the organism and enzyme production, which attained its maximum with the complete maturing of the fungus. Merrill and Clark, however, in their recent study of the gelatinase of *B. proteus-vulgaris*, reach the opposite conclusion, that growth, as evidenced by bacterial numbers, does not account for very significant differences in protease production which accompany variations in the salt content of the medium or in the oxygen available to the growing bacteria. It is their conclusion therefore that another factor, in the medium itself, is concerned with enzyme production.

PRESENT STUDY

The present study was begun as an investigation of protease production by *B. subtilis*, in the ordinary media, and in synthetic media, containing inorganic nitrogen and various carbon compounds. An investigation of *B. pyocyaneus* (*Pseudomonas aeruginosa*) was later undertaken because of conclusions reached as

to the relation of growth to protease production which necessitated the study of the number of viable bacteria in the cultures, and this could not be undertaken with a spore-bearing organism like *B. subtilis*.

A stock laboratory strain of *B. subtilis* was used, obtained from the Cambridge Biochemical Laboratory.

The following culture media was used:

(1) Tryptic broth
(2) 1 per cent gelatin in inorganic medium
(3) 1 per cent Witte's peptone in inorganic medium
(4) 1 per cent Witte's peptone in glucose inorganic medium
(5) 1 per cent lactic acid (as neutral salt) in inorganic medium
(6) 1 per cent glycerol in inorganic medium
(7) 1 per cent acetic acid (as neutral salt) in inorganic medium
(8) 1 per cent citric acid (as neutral salt) in inorganic medium
(9) 1 per cent tartaric acid (as neutral salt) in inorganic medium
(10) 0.1 tyrosine in an inorganic medium
(11) 1 per cent alanin in inorganic medium
(12) 1 per cent glucose in inorganic medium

The inorganic medium consisted of

	per cent
Dibasic ammonium phosphate	0.5
Acid potassium phosphate	0.1
Sodium chloride	0.1
Magnesium sulphate	0.025
Iron (as chlorid)	Trace

A stock solution equal to six times these values was made and diluted as needed.

The inorganic medium used with tyrosine contained no ammonium salt.

For the preparation of the lactate inorganic medium, an excess of its gram equivalent of sodium hydroxide was added to a weighed amount of pure lactic acid, and the solution, made up to a known volume with water, was heated in the autoclave at 15 pounds steam pressure for twenty minutes to hydrolyze the lactone. It was then brought to a pH of 7.4 with hydrochloric acid, using phenol red as an external indicator, and sufficient distilled water was added to make the final concentration of lactic acid 20 per cent. Stock 20 per cent solutions of acetate, tar-

trate and citrate were prepared by neutralizing known amounts of the acids with sodium hydroxide and making up to volume directly.

The broth, gelatin and peptone media were adjusted to a pH of 7.4 before sterilization in the autoclave to give a final pH of 7.2. It was found that for the synthetic media a pre-sterilization pH of 7.6 was needed to bring the final pH to 7.2.

Glucose in 20 per cent solution was sterilized separately and added at the time of making the cultures.

The cultures were made in 150 cc. of medium in Roux flasks of one litre capacity to provide ample surface for growth.

Cultures to be examined were first centrifuged. The supernatant liquid was adjusted to a pH of 7.4 and filtered through a Seitz asbestos filter. All apparatus was sterilized before use for twenty minutes at 115 pounds steam pressure. Cultures made from the filtrates were sterile.

Gelatin and casein solutions for testing the proteolytic activity of the filtrates were made as follows: Commercial gelatin to make a 5 per cent solution was dissolved, by heating in a water bath at 60°C. in phosphate buffer, pH 7.4, and 10 per cent alcoholic solution of thymol was added to make a concentration of 0.1 per cent. The gelatin was tubed in 5 cc. lots. Merck's casein to make a 1 per cent solution was dissolved by gentle rotation in buffered 0.9 per cent solution of sodium chloride. Thymol was added, as above, as a preservative. This constituted the stock casein solution. For use, the stock solution was diluted with buffered salt solution until a solution was obtained which, when further diluted to allow for the maximum amount of filtrate to be added, became opalescent when acidified with 0.2 cc. of 15 per cent acetic acid in 45 per cent alcohol (Diehl). The diluted casein was adjusted to a pH of 7.4 and was also tubed in 5 cc. lots.

The sterile filtrates were transferred to the test solutions in the following amounts, 0.2, 0.5, 1.0 and 2.0 cc. Control tubes contained 2.0 cc. of filtrate, but these tubes were plunged for five minutes into boiling water before being incubated. The tubes were incubated for 18 hours at 37°C. For examination, the gelatin

tubes were plunged into ice water for twenty minutes. Failure of the gelatin to harden was considered evidence of proteolysis. Casein digestion was indicated if no opalescence occurred after addition of 0.2 cc. of the acid alcohol solution. In the protocols of the experiments readings were made as follows, 0000 (control) indicated no digestion, and ++++, complete digestion.

MEDIA CONTAINING ORGANIC NITROGEN

Cultures were made upon three types of organic media. Two flasks of each type were used. Growth was rapid in all. One set was examined in four days and one in ten days. An active protease was found in all of the cultures.

SYNTHETIC MEDIA

For testing enzyme production in synthetic media, the organism was first sown in a lactate inorganic medium from a twenty-four hour tryptic broth-agar slant. It was subcultured twice into lactate inorganic medium before being inoculated into its final medium.

Lactate inorganic medium. Growth was noticeably slower in lactate inorganic medium than in tryptic broth, gelatin or peptone. A gelatin liquefying enzyme had appeared in three days. In four days an enzyme was present which was very active in the liquefaction of gelatin and feebly active for casein. The filtrate of the seven-day culture was very active for both proteins.

Glycerol inorganic medium. In glycerol the organism grew very rapidly. Four-day cultures were proteolytic, six-day cultures slightly more active.

Acetate, citrate and tartrate inorganic media. B. subtilis grew in none of these media. A very slight growth appeared in the acetate-inorganic flasks, but came to an apparent standstill after the second day. The hydrogen ion concentration of the media remained unchanged. Cultures made with added calcium carbonate to neutralize any acid which might be inhibiting growth likewise showed no growth. It was not surprising, therefore, that filtrates from the flasks showed no proteolytic activity whatever.

Glucose-inorganic medium. Growth in this medium was more

rapid than in lactate or glycerol and a protease could be demonstrated in cultures after three days. As glucose was the only source of carbon in this medium the ability of *B. subtilis* to use this sugar is assumed.

AMINO ACIDS

Cultures in tyrosine grew very little. Examined in eight days there was a change of pH to the alkaline side showing some utilization of the amino acid. Casein was very feebly attacked by filtrates of these cultures, but gelatin was perfectly liquefied in three tubes.

Cultures in alanin gave slightly more vigorous growth and a more active enzyme.

PROTEASE PRODUCTION IN THE PRESENCE OF AN ALTERNATE SOURCE OF CARBON

Cultures were made in Peptone (Witte) Inorganic Medium to which was added sterile 20 per cent glucose to make a final concentration of 1 per cent. Growth was poor in this medium. In four days the pH was neutral (7.0) and Fehling's solution was reduced. A gelatinase was present in the tube containing 0.5 cc. of filtrate and a caseinase in the tube containing 0.2 cc.

Comparison was also made of gelatinase production in a 1 per cent Difco peptone-1 per cent gelatin-inorganic medium and in the same medium to which was added glucose to make a concentration of 2 per cent. In three days the cultures were examined. Initial growth was more vigorous in all these flasks than in the Witte peptone. The pH was 8+ in the plain peptone-gelatin and 6.0 in the glucose peptone-gelatin. Copper was reduced by the latter culture. Gelatinase was present in both.

Gelatinase production was also demonstrated in a glucose-tryptic broth culture. The original glucose content of the medium was 2.53 per cent. In eighteen hours an active gelatinase was present and the glucose content of the medium was 0.66 per cent. This finding, together with the evidence of growth and protease production upon synthetic medium in which glucose

was the only source of carbon, removes any doubt of the ability of *B. subtilis* to utilize glucose or to produce protease while actively fermenting glucose.

From these experiments certain tentative conclusions were drawn:

1. It appeared in the first place that a fairly vigorous growth must take place before a proteolytic enzyme is demonstrable. This would explain the appearance of protease in younger cultures in the protein media than in synthetic media in which the lag period is greater, and its decrease or complete absence in media in which growth is very poor or is altogether inhibited.

2. A protease can be elaborated by *B. subtilis* in the absence of organic nitrogen.

3. The hypothesis of Diehl concerning the specificity of protease for amino-acids is not confirmed.

4. The addition of a fermentable sugar in a concentration of 1 or 2 per cent does not appear to affect protease formation by *B. subtilis*, although active utilization of glucose can be demonstrated.

The study of a second proteolytic organism, *B. pyocyaneus* (*Pseudomonas aeruginosa*) was then undertaken in order to observe the number of viable bacteria in relation to the proteolytic activity of the cultures. It was desired to study also *B. proteus-vulgaris*, but, of several strains examined, none were found to grow readily in synthetic media and the study was therefore confined to *B. pyocyaneus*. Two strains of this organism have been studied,—*B. pyocyaneus 254*, from the Collection of Type Cultures, Lister Institute, London, and *B. pyocyaneus T.*, isolated from a case at the Philadelphia General Hospital.

Several changes of procedure were adopted. The amount of enzyme present was determined by observing the time required to bring about proteolytic change, that is for the liquefaction of gelatin or for rendering casein acid-soluble. Liquefaction of gelatin within one-half hour was designated as "very good," within four hours "good," and within twenty-four hours "fair." Incomplete liquefaction, but a noticeable increase in the hardening time over that of a control gelatin solution containing no enzyme,

was designated as a "trace" of gelatinase. The same principle was applied to the determination of proteolytic action upon casein A 6.5 per cent Difco gelatin in slightly buffered water and preserved with 0.5 per cent phenol was accordingly prepared for each set of tests. To 20 cc. of this solution were added 5 cc. of the culture to be tested. This was incubated at 37.5°C. Two-cubic-centimeter samples were withdrawn at intervals and adjusted to a pH of 6.2 with N/20 hydrochloric acid. The tubes were then plunged into ice water and the time required for the gelatin to harden counted in minutes. The control setting time was determined on a tube of 5 per cent gelatin at pH 6.2. The method was modified from that of Clark for determining the activity coefficient of protease solutions.

Casein was prepared as outlined above, Pfanstiehl's casein being substituted for the Merck preparation, and 0.5 per cent phenol being used as a preservative. Twenty cubic centimeters of the dilute casein were incubated with 5 cc. of the bacteria free filtrate.

It was found that the optimum pH of *B. pyocyaneus* proteus for gelatin was about 7.4, while that for casein was more on the alkaline side, about 7.8 to 8.2. This follows the findings of Northrop in relation to variation of optimum pH with substrate.

The gelatin-enzyme mixtures were therefore incubated at a pH of 7.4 and the casein enzyme mixtures at a pH of 8.0.

Viable counts were made by Wilson's modification of the Esmarch roll-tube method.

GROWTH AND PROTEASE PRODUCTION BY B. PYOCYANEUS (PSEUDOMONAS AERUGINOSA) IN PROTEIN MEDIA

Gelatinase production was first studied in tryptic broth. 40,000,000 organisms of a three-hour culture of *B. pyocyaneus T.* in tryptic broth were inoculated into 100 cc. of tryptic broth (pH 7.2) in a 1000 cc. Roux flask and incubated at 37.5°C. In eighteen hours the viable count had risen to 1,750,000,000 and the inactivated culture liquefied gelatin after 11 minutes incubation. Two similar cultures of *B. pyocyaneus 254* were made. The initial number of bacteria in one was very small and maximum

growth was therefore not reached in twenty-four hours. In forty-eight hours the number of viable bacteria exceeded 1,000,000,000 per cubic centimeter and a very active enzyme for both gelatin and casein was present. Further experiments on growth in tryptic broth, peptone media and peptone-gelatin media, are reported below in connection with experiments on the effect of added carbon compounds upon protease production.

Protease production was studied in protein media to which was added glucose in concentrations of 0.5, 1.0 and 2.0 per cent. Table 1 summarizes the gelatinase production in a medium of peptone made as follows:

	per cent
Peptone (Difco)	1
Na_2HPO_4	1
KH_2PO_4	0.2
NaCl	0.2
$MgSO_4$	0.05
$CaCO_3$	0.05

One hundred cubic centimeters of this medium in Roux flasks of 1000 cc. capacity (pH 4) were sterilized in the autoclave. One flask served as a control and to the three others were added 20 per cent glucose solution, sterilized by passage through the Seitz filter, in amount sufficient to make the desired concentration. At the same time a similar set of flasks containing one per cent Difco peptone in 0.2 per cent saline without added buffer or salts was prepared. These are designated as peptone unbuffered and peptone buffered.

Initial growth was poor in all, except the buffered 1 per cent glucose peptone. The latter was the only culture in which gelatinase activity was *Good*. The cultures in the glucose-containing media were all more proteolytic than those in the plain peptone. The buffered cultures showed slightly greater initial growth than the unbuffered, but neither showed vigorous growth in the first twenty-four hours.

The experiment was repeated with another strain of the organism, using tryptic broth as the protein medium. The results are summarized in table 2. A *Very Good* gelatinase and caseinase

TABLE 1

Gelatinase production by Pseudomonas pyocyaneus T. in peptone media with added glucose

MEDIUM	AGE	pH	VIABLE ORGANISMS	GELATINASE
	hours		*millions per cu. cm.*	
Peptone control	24		172.5	Trace
	96	8.4	12.5	Trace
Unbuffered peptone with glucose 0.5 per cent	24		166.2	Trace
	96	8.4	138	Fair
Buffered peptone with glucose 0.5 per cent	24		171.2	Trace
	96	8.4	105	Fair
Unbuffered peptone with glucose 1 per cent	24		280	Trace
	96	7.8	217.5	Fair
Buffered peptone with glucose 1 per cent	24		417	Trace
	96	7.0	215	Good
Unbuffered peptone with glucose 2 per cent	24		87.5	Trace
	96	6.6	50	Fair
Buffered peptone with glucose 2 per cent	24		220	Trace
	96	6.6	95	Fair

TABLE 2

Gelatinase production by Pseudomonas pyocyaneus 254 in tryptic broth with increasing concentrations of glucose

MEDIUM	AGE	REDUCTION COPPER	VIABLE ORGANISMS	GELATINASE	CASEINASE
	hours		*millions per cu. cm.*		
Plain tryptic broth	18	0	650	Fair	
	48	0	1,900	Very good	Very good
1 per cent glucose tryptic broth	18	+	105	Fair	
	42	+	600	Fair	None
	66	+	10	Very good	Very good
2 per cent glucose tryptic broth	24	+	12.5	None	
	48	+	110	Fair	Fair
2 per cent glucose tryptic broth $CaCO_3$ 0.025 per cent	24	+	98	None	
	48	+	740	Very good	Very good

were found in all the cultures, except one, that in 2 per cent plain glucose-tryptic broth. Growth was poor in this medium. The very active protease in 2 per cent glucose broth to which was added 0.025 gram of calcium carbonate would indicate that the fermentation of glucose could not of itself account for the less active enzyme.

GROWTH AND PROTEASE PRODUCTION IN SYNTHETIC MEDIA

Synthetic Medium I contained dibasic ammonium phosphate (9.5 per cent), monobasic potassium phosphate (0.1 per cent) and sodium chloride (0.1 per cent) as in the inorganic medium described above. Glucose in 0.025 per cent concentration was used as a source of carbon. The glucose was sterilized by passage through the Seitz filter and was added with a sterile pipette at the time of making the cultures. Salts of magnesium, calcium and iron were added in the amounts indicated in table 3, so that a series of cultures in a medium having the same basic constituents, but varied as to its salt content could be observed for rate of growth and for protease production. The cultures were made in Roux flasks of 1-liter capacity, each containing 100 cc. of the medium at pH 7.2. Only a *trace* of gelatinase was found in the flask containing the basis medium, and in that containing only a trace of iron in addition to the basic medium. Growth was poor in both these flasks. Growth was slightly better and a *fair* enzyme production was attained in the flask in which magnesium sulphate was added to the iron. "Good" enzyme production was observed in the flasks containing magnesium, and calcium both alone and in combination, but no significant differences of growth or protease production were observed in this set of cultures between the cultures in magnesium alone, calcium alone or the two in combination.

A second synthetic medium was prepared as above, but sodium lactate (1 per cent) and glycerol (0.5 per cent) were substituted for the glucose as sources of carbon. Magnesium sulphate and calcium carbonate were added as indicated in table 4. In this series of cultures two concentrations of each salt were used, so that any variation in growth or protease production due to varia-

tion in salt concentration might be observed. The difference in both growth and protease production in the flasks containing magnesium sulphate is very striking. In the concentrations of calcium carbonate, on the other hand, neither growth nor protease production equalled those observed in the first series, when

TABLE 3

Gelatinase production by Pseudomonas pyocyaneus T. in synthetic medium I with varying salt additions

SALT ADDED, CONCENTRATION	AGE	VIABLE ORGANISMS	GELATINASE
	days	*millions per cu. cm.*	
None	2	58.3	None
	4	125.0	Trace
	12	72.5	Trace
$MgSO^4$ 0.025 per cent	2	257.0	Fair
	4	530.0	Good
	12	185.0	Good
$CaCO_3$ 0.025 per cent	2	420.0	Fair
	4	601.0	Good
	12	92.5	Good
$MgSO^4$ 0.025 per cent, $CaCO_3$ 0.025 per cent	2	392.0	Fair
	4	467.0	Good
	12	146.3	Good
FeCl (trace)	4	99	Trace
	8	225	Trace
$MgSO^4$ 0.025 per cent FeCl (trace)	2	390	Fair
	6	267.4	Fair
$MgSO^4$ 0.025 per cent, $CaCO_3$ 0.025 per cent, FeCl (trace)	2	270	Fair
	4	360	Good
	12	132.5	Good

a concentration of calcium carbonate, intermediate between those in this series, was used. It will be noted that, both in bacterial numbers and in the amount of enzyme present, all the cultures of this series containing magnesium equalled the cultures in tryptic broth.

TABLE 4

Protease production by Pseudomonas pyocyaneus 254 in synthetic medium 2 with varying concentrations of magnesium and calcium

ADDED SALT	pH	VIABLE ORGANISMS	GELATINASE	CASEINASE
		millions per cu. cm.		
None	8.2	75	None	None
$MgSO_4$ 0.0125 per cent	8.6	715	Very good	Good
$MgSO_4$ 0.05 per cent	8.6	307.7	Fair	Fair
$CaCO_3$ 0.0125 per cent	8.6	206	Fair	Trace
$CaCO_3$ 0.05 per cent	8.6	176	Fair	None
$MgSO_4$ 0.0125 per cent, $CaCO_3$ 0.0125 per cent	8.6	1,145	Very good	Good
$MgSO_4$ 0.05 per cent, $CaCO_3$ 0.05 per cent	8.6	761	Very good	Very good

TABLE 5

Comparison of protease production in duplicate cultures of Pseudomonas pyocyaneus 254 in synthetic medium 2 with magnesium alone and magnesium and calcium combined

CONCENTRATION OF ADDED SALT	AGE	VIABLE ORGANISMS	GELATINASE	CASEINASE
	hours	*millions per cu. cm.*		
$MgSO_4$ 0.025 per cent	22	159	Trace	
	48	511	Fair	
	72	619	Good	Good
	96	217	Good	Good
$MgSO_4$ 0.025 per cent	22	166.8	Trace	
	48	549	Fair	
	72	292	Good	Good
	96	190	Good	Good
$MgSO_4$ 0.025 per cent, $CaCO_3$ 0.025 per cent	22	133.8	Trace	
	48	768	Good	
	72	610	Good	Good
	96	326	Good	Good
$MgSO_4$ 0.025 per cent, $CaCO_3$ 0.025 per cent	22	86.6	Trace	
	48	797	Good	
	72	590	Good	Good
	96	432	Good	Good

Cultures of the same strain of *B. pyocyaneus* in synthetic medium 2 to which were added magnesium sulphate and calcium carbonate, in a concentration of 0.025 per cent as used in cultures of *B. pyocyaneus T.* (see table 3) were next studied (table 5). The results were comparable with those for *B. pyocyaneus T.* in the same salt concentrations, and could be duplicated.

From the foregoing it is obvious that an active proteolytic enzyme can be elaborated by *B. pyocyaneus* in a synthetic medium containing no organic nitrogen. Indeed, under suitable conditions of salt concentration, this enzyme may practically equal in activity that produced in tryptic broth. As Merrill and Clark have observed, however, no more than a "trace" of protease is to be found in any synthetic medium in the absence of the salts of magnesium or calcium. The part played by these salts in protease formation is considered by these investigators to be independent of the part they play in stimulating growth. This conception of the rôle of magnesium and calcium in protease production the author cannot, on the basis of her own findings, accept for several reasons.

In the first place, as will be seen from a review of tables 3 to 5 both growth and protease production may be made to vary in amount by varying the salt content of the medium. Further, a protease is not demonstrable even under the best of conditions in synthetic medium, or in any medium whatever, until sufficient growth has occurred. If the maximum bacterial numbers observed in cultures in synthetic media containing no magnesium or calcium are compared with the number of viable bacteria in cultures in the same medium, but containing in addition magnesium or calcium, at an early stage of growth when not more than a trace of protease is demonstrable, it will be seen that the bacterial population of one is comparable with that of the other.

In following the course of many cultures it will be seen that, as the number of viable bacteria increases, proteolytic activity also increases until maximum growth is attained. After this point is reached the viable bacterial population steadily declines but proteolytic activity remains stationary. Maximum proteolytic activity in other words will correspond only to maximum growth.

The only valid comparison that can be made between maximum enzyme activity and growth must accordingly be based upon the maximum and not upon the final number of viable organisms observed.

The relation between growth and enzyme activity up to the point of maximum growth is not a straight line relationship. If this were so it should be possible to decrease the activity of any "good" culture, for example, to that of a "fair" culture or one containing only a "trace" of protease by so diluting the former that its bacterial population corresponded to that of one of the latter. Dilution experiments of this sort were made and showed that to reduce a fresh culture from "good" to "fair" enzyme activity when the proportion of population to population, i.e., of a "good" culture compared with a "fair," was roughly 2:1, required at least a dilution of 1:50, while to reduce an old culture from "good" to a "trace" required a dilution of 1:75. It can be readily seen therefore that, after protease becomes demonstrable, no real increase in growth is to be regarded as insignificant and that, on the other hand, differences in numbers between cultures of unequal activity need not be striking.

A final experiment was made to determine the mechanism of bacterial protease production. The observation that increase in activity could on occasion be accompanied by a fall in the viable bacterial count suggested that autolysis might be a factor in protease production. A preparation of autolysed cells was accordingly made from an actively proteolytic culture of *B. pyocyaneus 254* in synthetic medium 2, containing 0.0125 per cent magnesium sulphate. The culture was first inactivated by the addition of phenol to make 0.5 per cent concentration, and was then centrifuged and the cells washed three times with slightly buffered water. They were then suspended in a phosphate buffer (pH 7.4). A portion of the cell suspension was set aside for further experiment with the intact cells. The remainder of the suspension was allowed to autolyze under toluol in the incubator at 37.5° for three days. The preparation was centrifuged and the supernatant fluid showed no proteolytic activity after twenty-four hours incubation with gelatin. The suspension of intact cells, on the

other hand, liquefied gelatin in twenty-four hours. The latter observation was repeated upon the cells of *B. subtilis*, and upon cells from another culture of *B. pyocyaneus* in a synthetic medium. The results of these experiments would indicate that the cellular phase alone is concerned with protease production and that the intact bacterial cell is the source of protease. This observation falls in line with those of Cook and Stephenson for other bacterial enzymes, the dehydrogenases. These investigators found that exposure to ultra violet light, sufficient to prevent further growth, did not interfere with the oxidations activated by *B. coli*.

SUMMARY

Protease production by *B. pyocyaneus* has been found to be roughly parallel with growth. The time of appearance of a proteolytic enzyme varies therefore in relation to the rate of growth. In the vigorously growing cultures in tryptic broth in which maximum growth is attained in from twenty-four to forty-eight hours, as a rule, the maximum enzyme activity appears so ner than in cultures in peptone or in the synthetic media in which growth is delayed.

Variations in protease production in a series of cultures made in buffered synthetic media of the same basis composition, that is containing the same sources of nitrogen and carbon, and varied only in relation to their salt content, appear to be explicable solely on the basis of variations in growth. No particular salt was found to have a stimulating effect upon enzyme production independent of its growth stimulating effect. In the cultures in synthetic media in which maximum bacterial numbers approached those in the protein media an equally active protease was observed.

It has again been demonstrated that the presence of an alternative source of carbon has no effect in delaying the appearance of a protease, a highly active enzyme appearing in cultures containing a carbohydrate readily available to the organism in a concentration greatly in excess of needs.

Confirmation is again given to the observation that a protease may be as readily elaborated from inorganic nitrogen as from

organic nitrogenous compounds, and in a suitable medium may equal in activity that found under the most favorable conditions.

Protease appears to to be a product of the intact bacterial cell.

CONCLUSIONS

Protease production by the organisms studied is a process of ordinary bacterial cell metabolism and is not dependent upon the ingredients of the nutrient medium, except as these stimulate or inhibit cell growth.

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A COMPARATIVE STUDY OF MEMBERS OF THE LACTOBACILLUS GENUS, WITH SPECIAL EMPHASIS ON LACTOBACILLI OF SOIL AND GRAIN[1]

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Although lactic acid organisms were among the first to be studied, and though their isolation and their action on carbohydrates represent some of the first chapters in the history of bacteriology, they are still a much discussed and much disputed group. They have been described and classified in many ways by different workers. No group of lactic acid organisms shares in this controversy more than the lactobacilli.

Workers have been divided in their views. Some maintained that organisms of this genus, Lactobacillus, were not sufficiently distinct to warrant division into several species. Others have designated new species, on the basis of only very slight variations in morphology and cultural reactions.

Of the investigators who believed that the differences found are insufficient to divide the group into species, Rodella (1901 and 1908) and Heinemann and Hefferan (1909) may be cited. Each emphasized the pleomorphic nature of the Lactobacillus group and called attention to the presence of long rods and coccoid forms in the same culture. Heinemann and Hefferan concluded that these organisms had not been investigated thoroughly under uniform conditions, and that when so studied the differences were slight. They gave a composite description based upon a study of several organisms. They contended that

[1] This paper covers in part the work submitted to the Graduate School of Yale University by the senior author as part requirement for the degree of Doctor of Philosophy.

the Bacillus of Massol, *B. acidophilus*, the Boas-Oppler bacillus, *B. panis-fermentati*, *Streptobacillus lebenis*, and *Leptothrix buccalis* were synonyms for one and the same organism, *B. bulgaricus*.

Henneberg (1904) and Rahe (1914 and 1918) held other views. Henneberg, working with twenty-two strains, made an extensive morphological and cultural study of this group. The information contained in his orginal paper (*Zeitschrift für Spiritusindustrie*) formed the basis for many subsequent systems of classification. He divided *L. leichmanni* into 3 sub-groups according to the fermentation reactions on 14 carbohydrates.

Rahe divided his strains of *Lactobacillus acidophilus* into four sub-types, *L. acidophil-aerogenes* into eight, and *L. bulgaricus* into four sub-types. *L. bifidus* he did not subdivide, but classified as an undivided species. The differentiation was established entirely on the basis of fermentation reactions of five carbohydrates and of mannitol.

The difficulty encountered in establishing significant distinguishing characteristics is reflected in the literature in many instances. When a lactic acid organism was encountered as a contaminant in butyl alcohol and acetone fermentation, Thaysen (1921) believed it to be a new species and called it *B. volutans*. Later, work by Fred, Peterson and Stiles (1925) showed it to be a member of the Lactobacillus group and probably *L. leichmanni*.

Howe and Hatch (1917) isolated organisms of the Lactobacillus group from carious teeth and referred to them as *L. acidophilus* and *L. bifidus*. Rodriguez (1922), in a study of aciduric organisms isolated from deep foci of active dental caries, suggested that they be called *B. odontolyticus* (types I, II and III). McIntosh, James and Lazarus-Barlow (1922) found two types of organisms which they named *B. acidophilus-odontolyticus* (types I and II). Bunting, Nickerson and Hall (1926) called the organism isolated by them from caries, *B. acidophilus*. Morishita (1928, 1929) has noted that the organisms isolated by himself and others from carious teeth are, with few exceptions, not *L. acidophilus* as this species or type has been described by Kulp and Rettger (1924), and by others.

These scattered examples are but a few of many such instances.

Inasmuch as several investigations[2] made in this laboratory have dealt with organisms of the Lactobacillus genus found in the intestinal tract and in dental caries, it was thought advisable to make a study of some of the Lactobacillus organisms found in soil and grain, with particular emphasis on those which produce relatively large amounts of lactic acid from carbohydrates, and which bear such an important relation to lactic acid production as a commercial biological process.

The present paper covers one phase of an investigation conducted by the writers on *Biological Production of Lactic Acid by High Acid-producing Organisms of the Lactobacillus Genus.*[3] The 36 strains of Lactobacillus described here were obtained from the following sources: 18 isolated by the writers from soil, grain and fecal material; 9 from the American Type Culture Collection; 3 from the University of Wisconsin; 3 from Morishita's collection of dental organisms; and 3 from the laboratory stock of *L. acidophilus* and *L. bulgaricus*. A fuller account of the sources follows.

Sources of Lactobacillus strains

Numbers	*Authors' isolations*
11, 12, 13, 15	Lactobacilli isolated from drains of Yale University Armory.
23, 24, 28, 29	Lactobacilli isolated from stalls of Yale University Armory.
32, 34	Lactobacilli isolated from soil.
71, 72, 73	Lactobacilli isolated from bran.
85, 87	Lactobacilli isolated from cess pool.

[2] Rettger and Horton, (1914); Hull and Rettger, (1917); Rettger and Cheplin, (1920); Kulp and Rettger, (1924); Morishita, (1928, 1929).

[3] Work still in progress.

A7—Lactobacillus resembling A2 (see below) at time of isolation from a sample of barley. This culture was carried as a stock culture for two years before fermentation reactions described in this paper were determined.

A8—Lactobacillus resembling A4 (see below) at time of isolation from sample of corn meal. Strain carried as a stock culture for two years before fermentation reactions described in this paper were determined.

Alf—Lactobacillus isolated from alfalfa meal.

From the American Type Culture Collection:

A1—*Lactobacillus leichmanni* (368).
A2—*Lactobacillus delbrücki* (899).
A3—*Lactobacillus lycopersici* (4005).
A4—*Lactobacillus fermentatae* (4006).
A5—*Lactobacillus acidophil-aerogenes* (4007).
A6—*Lactobacillus acidophil-aerogenes* (4009).
A9—*Lactobacillus delbrücki* (4172).
A10—*Lactobacillus pentoaceticus* (367).
A11—*Lactobacillus plantari* (4377).

From Dr. Fred, University of Wisconsin:

F1—*Lactobacillus leichmanni* (isolated from corn meal).
F2—*Lactobacillus leichmanni* (isolated from corn mash).
F3—*Lactobacillus delbrücki* (Henneberg).

From Morishita, Yale University:

T1—*Lactobacillus odontolyticus* (33).
T2—*Lactobacillus odontolyticus* (19).
T3—*Lactobacillus odontolyticus* (BN).

From Yale University:

Laboratory of General Bacteriology:

RH—*Lactobacillus acidophilus*
K1—*Lactobacillus acidophilus* (Scavano).
K2—*Lactobacillus bulgaricus* (B-12).

All strains were Gram-positive rods occurring singly, in pairs or in chains. They varied greatly in size, at times even in the same culture (0.3 to 1.0 by 1.0 to 20μ). They were microaerophilic and fermented carbohydrates and alcohols with the production of lactic acid and varying amounts of acetic acid. Some of them formed two types of colonies in agar, described by Rettger and Horton (1914) as x and y types. The descriptions correspond with that given by Fred, Peterson and Stiles (1925) for lactic acid organisms isolated from soil (table 2).

ISOLATION OF LACTOBACILLUS STRAINS

The method of isolation was a modification of that used by other workers, namely the use of acid enrichment broth containing

a fermentable carbohydrate. However, in working with soil and grain as a source of organisms, much difficulty was encountered with fungi overgrowing the lactobacilli in the enrichment medium and in the plates which were subsequently poured. The following procedure was adopted with very satisfactory results.

Medium used:

Corn flour	50 grams
Peptone	10 grams
Water	1000 cc.

The mixture was boiled for fifteen minutes and adjusted to a reaction of pH 3.5 to 4.0 with lactic acid. The medium was dispensed in 10 cc. portions in test tubes, and sterilized for twenty minutes at 15 pounds extra pressure.

The tubes were inoculated with samples of grain or soil weighing about 1 gram, or with other test material in liberal amounts; a layer of sterile mineral oil 10 to 15 mm. thick was poured over the mash and the tubes incubated at 37 or 40°C. for forty-eight or seventy-two hours. Plates poured from these tubes often contained only lactobacilli in the higher dilutions. Molds were seldom seen on any of the plates.

The colonies selected for isolation were examined under the low power of the microscope and cut out of the agar by a sterile platinum spatula prepared by flattening a platinum wire slightly at one end. The agar block containing the colony was crushed in a tube of broth and immediately replated. Colonies selected from the second plating usually gave pure cultures. In some instances single cells were isolated by the improved Barber micropipette method for single cell culture study. For a full description of the technique employed here see Gee and Hunt (1928).

Several media were used for cultivation and preservation of stock strains. Yeast-water broth and agar containing glucose and peptone were found very favorable; they were prepared as follows: One pound of compressed yeast was added to 4 liters of water and suspended by shaking. Twenty-five cubic centimeters of chloroform were added to prevent bacterial growth. The

flask was incubated for forty-eight hours at 37°, the mixture being thoroughly shaken at intervals. It was then steamed in the autoclave until the chloroform had volatilized, and finally heated at 15 pounds pressure for an hour. After the yeast cells were allowed to settle, the supernatant fluid was removed, and clarified with egg albumin when this was found to be necessary. The resultant yeast water should have a clear amber or light brown color. To attain this end quickly the soluble starch which slowly precipitates and settles upon standing may be changed by the addition of a small amount of Takadiastase or filtered saliva when the yeast water is still warm.

The yeast water was made into the following medium:

Glucose	10 grams
Peptone	10 grams
Yeast water	300 cc.
Water	to 1000 cc.

This medium was adjusted to pH 6.8, tubed and sterilized in the autoclave.

Tomato juice broth as devised by Kulp (1927) was found to be almost as good as yeast water for some strains and better for others. It is prepared more easily than the other, and was used in many instances in this work to supplement the yeast water medium.

For the preservation of stock strains the following medium has many advantages:

Skimmed milk	1000 cc.
Peptone	10 grams
Glucose	10 grams
Litmus (enough to give a deep blue color).	

This was dispensed in test tubes containing about 0.5 gram of calcium carbonate per tube. The tubes were plugged and sterilized at 15 pounds for twenty minutes in the autoclave. When the seeded tubes were protected from excessive drying the organisms remained viable for at least seven months without apparent change of fermentation reactions. However, stock cultures were transferred at least once a month as a routine procedure.

With the use of the isolation technic described and with the yeast water and tomato juice media it was possible to isolate aciduric organisms of the Lactobacillus type from cracked wheat, rye meal, cracked corn, oats, corn flour, alfalfa meal, barley, bone meal, meat scraps, chicken feed and rolled oats. These samples represented grains purchased in a variety of wholesale and retail food stores and cereal mills located in the Eastern and Mid-Western sections of the United States.

Fifty samples of recently collected soil representing all but two types of surface soil found in Linn County, Iowa, were examined.[4] Organisms of the Lactobacillus group were present in all of these samples.

Samples of soil or grain kept in the laboratory in test tubes or bottles rapidly lost their viable lactobacilli. In fact, none of the fifty samples of soil contained viable lactic acid organisms of this type two months after the date of collection. However, lactobacilli were readily demonstrated in samples of grain which had been held in a feed store bin for several weeks or in a paper bag in the laboratory store room. In such instances mouse feces were found to be present in the samples, and there was ample evidence that mice had been feeding on the grain. In one instance a bag of alfalfa meal remained on a shelf in a small store room or closet for several months during which time it was a source of food for mice, as was clearly indicated by the scattered meal on the shelf. A sample of this exposed meal revealed aciduric organisms, while a similar sample protected from mice by a wire screen showed none after weeks of storage.

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS

Morphological and cultural characteristics are shown in table 1. Tubes of glucose yeast-water broth were inoculated with the suspension of the washed organisms and incubated at one of four temperatures, 42, 37, 16 and 12°C., for forty-eight hours. The cultures were also plated out in glucose yeast-water agar; tubes of glucose yeast-water gelatine were inoculated and in-

[4] These were made available through the kindness of Prof. Anderson, Coe College.

TABLE 1

Giving certain morphological and cultural characteristics

CULTURE	MORPHOLOGY	COLONY FORMATION ON GLUCOSE YEAST WATER AGAR	GROWTH IN GLUCOSE YEAST WATER BROTH	GROWTH IN GLUCOSE YEAST WATER GELATIN AT 22°C.	pH AT 42° INCUBATION (GLUCOSE YEAST WATER BROTH)	pH AT 37° INCUBATION	pH AT 16° INCUBATION	pH AT 12° INCUBATION
A1	Short thick rod	x	MT.S	+	3.3	3.3	5.5	7.0
A2	Short thick rod in chains	x and y	C.S	3+	3.3	3.5	4.5	6.0
A3	Long rod single or pairs	x	MT.	+	5.5	6.0	6.0	7.0
A4	Short to long rods	y	ST.	3+	4.0	4.5	6.0	6.8
A5	Short rod in pairs	x and y	MT.S	4+	3.6	3.4	5.0	5.5
A6	Short rod in pairs	x and y	MT.S	4+	3.3	3.3	4.8	6.6
A7	Short rod streptococci-like chains	y	C.S	4+	4.4	4.6	6.0	7.0
A8	Short to long rod, single or in pairs	y	ST.S	3+	4.6	4.6	5.5	6.5
A9	Long rods, single or in pairs	x	C.S	0	4.0	4.4	7.0	7.0
A10	Very small rod, single or in pairs	y	ST	0	4.4	4.4	6.6	7.0
A11	Short rod in short chains	x	C.S	0	4.0	4.4	7.0	7.0
F1	Short to long rod	x and y	MT.S	0	4.0	4.0	7.0	7.0
F2	Short to long rod	x and y	MT.S	0	3.4	3.8	7.0	7.0
F3	Short rod in short chains	x and y	C.S	3+	3.4	3.4	4.5	5.5
Alf	Medium to short rod	y	ST	−	4.4	4.6	7.0	7.0
11	Medium rod, palisade-like growth	y	C.S	0	4.0	4.0	7.0	7.0
12	Short rod in pairs	x and y	C.S	0	3.4	3.8	7.0	7.0
13	Medium rod, palisade-like growth	y	C.S	0	3.6	4.0	7.0	7.0
15	Short rods in pairs	y	C.S	0	3.8	3.8	7.0	7.0
23	Short medium rod, occurring singly	y	C.S	0	5.0	4.6	7.0	7.0
24	Short thick rod in chains	x and y	C.S	0	4.6	4.6	7.0	7.0
28	Medium rod, palisade-like growth	x and y	C.S	0	4.6	4.4	7.0	7.0
29	Medium rod	y	C.S	0	4.0	4.0	7.0	7.0
32	Short medium rod, single or chains	y	ST	0	5.0	5.0	7.0	7.0
34	Medium rod	y	C.S	0	4.6	4.6	7.0	7.0
72	Medium rod palisade-like growth	x and y	C.S	0	3.4	3.8	7.0	7.0
85	Medium delicate rod	y	MT	0	3.4	4.2	7.0	7.0
87	Short to medium rod	y	ST.S	0	4.0	4.6	7.0	7.0

Key to table: ST = slight turbidity; MT = medium turbidity; C = clear; S = sediment.

cubated at 22°C. The acid production for the four temperatures of incubation, the type of colony in the agar, the growth in the gelatine, the appearance of growth in the broth, and the morphology of the organism found in an eighteen- to twenty-four-hour glucose yeast-water broth culture (prepared from stock cultures of the litmus milk containing glucose and calcium carbonate) are indicated in the table (1).

There were 4 *x* and 14 *y* types which remained reasonably constant, while 10 of the total number of 28 strains produced colonies which varied more or less between the *x* and *y* forms.

Nine members of the series produced visible growth in glucose yeast water gelatin at 22°C., whereas 18 refused to do so. All caused pronounced acid production in glucose yeast water broth at 37 and 42°, and 9 appreciable quantities at 16°C.; 19 failed to change the H-ion concentration of the medium at this temperature. Only three depressed the pH of the glucose broth at 12°.

Various writers have described members of the Lactobacillus genus as being extremely pleomorphic, the morphology of the individual strains varying from a distinct coccus form to long and short bacilli of extremely variable thickness. Such claims have recently been made by Rosebury, Linton and Buchbinder (1929). In some earlier publications of Rettger and his colleagues attention was called to very marked pleomorphism in the Bulgaricus-acidophilus group. More recent and intensive studies in our laboratory have shown, however, that under controlled uniform conditions of temperature and cultural environment the different strains are as a rule far less pleomorphic than has been so often claimed.

A certain amount of pleomorphism and cultural variation has been observed in the present investigation, and in some instances these seemed to vary with slight variation in environmental conditions, such as temperature, and with the intervals elapsing between transfers.

Key to table: ST = slight turbidity; MT = medium turbidity; C = clear; S = sediment.

FERMENTATION STUDIES

Casein-digest broth (although not as favorable a medium for many strains of Lactobacilli as yeast water or tomato broth) was

used. Prepared from C. P. casein by the tryptic digestion method described by Kulp and Rettger (1924), it is free from carbohydrates and promotes growth of all strains in varying degress when a fermentable carbohydrate is added.

Two media were made from this for the determination of fermentation reactions. One was casein digest broth which was sterilized before the adition of the carbohydrate. The carbohydrate was added in the form of a sterile aqueous solution. The second medium was a casein-digest soft agar containing Andradè's indicator:

Casein-digest	equivalent to 10 grams casein
Peptone	10 grams
Agar	5 grams
Andrade's indicator	10 cc.
Water	to 1000 cc.

Here again the carbohydrates were added as sterile aqueous solutions after the medium had been tubed, plugged and sterilized in the autoclave. Xylose and arabinose solutions were sterilized by Berkefeld filtration; all of the others by autoclaving. The concentration of carbohydrate used was 0.5 per cent in both the broth and the soft agar media.

The inoculum was prepared from a twenty-four hours yeast water or tomato juice broth culture by centrifuging the culture, removing the supernatant fluid and washing by resuspending and centrifuging the residual cells three times in sterile saline solution (0.85 per cent). A final saline suspension was prepared having a turbidity of 8 or 10 on the McFarland nephelometer scale. Plates were then poured to determine the purity of the suspension and 0.1 cc. of the suspension inoculated into casein digest sugar broth (3 to 5 cc. volume); a stab inoculation was likewise made in Andrade soft agar medium, using a 4 mm. loopful of the suspension.

The Andrade soft agar was used in making simple fermentation tests with organisms which were supposedly of the Lactobacillus type, immediately after their isolation from the corn mash medium. The inoculum was washed in sterile saline, as described above. The carbohydrates employed in these tests were glucose, lactose,

maltose, sucrose and xylose. A carbohydrate-free control was also employed.

The casein-digest broth containing 0.5 per cent of a given carbohydrate and inoculated with the washed culture, was incubated at 37° for three days and the pH determined by the colorimetric method. A decrease in pH above any change observed in the sugar-free control was taken as an indication of carbohydrate utilization. In some instances growth took place without any decrease in the pH being observed. When possible in these cases, the reducing sugar was determined by the Somogyi (1926) modification of the Shaffer-Hartmann reagent. In no such instance did a quantitative determination of the reducing sugar reveal a loss in carbohydrate.

The following system is used in table 2 to indicate ranges of pH.

− = no change, pH 7.0
±? = doubtful (pH 6.6 to 6.7)
± = pH 6.5 to 6.1
\+ = pH 6.0 to 5.6
++ = pH 5.5 to 5.1
+++ = pH 5.0 to 4.6
++++ = pH 4.5 or less.

The fermentation reactions were obtained in casein digest broth containing 0.5 per cent of the known carbohydrates and inoculated with washed cultures of the various strains.

An examination of the reactions in table 2 reveals several points of interest. A2, A5, A6, A7 and F3 resemble each other, although they are not identical. For instance, A2 and F3 slightly affect sucrose, while the others do not. F3 is the only one of these five strains which attacks dextrin, though this action is again slight. A2 and F3 act on mannitol more readily than do A5, A6 and A7. A5 and A6 are the only strains of these five which produce a visible change in milk.

A8 and A10 have some resemblences. A10 ferments raffinose, which is not acted upon by A8, but the latter ferments mannitol, salicin and milk, while A10 does not.

Although A1, F1 and F2 are all *L. leichmanni* strains, A1 differs from F1 and F2 in that it acts on lactose and salicin, as

well as on milk, although slowly. Except for the amount of acid produced, F1 and F2 are alike in their action.

A2, A9 and F3 are all *L. delbrücki* cultures, and although A2 and F3 resemble each other quite closely, A9 is different. A9 acts on lactose slightly, but does not ferment sucrose at all. It does not ferment melezitose or mannitol, and attacks raffinose feebly, while the other two are very active. A9 affects starch and milk,

TABLE 2

Showing fermentation re

CARBOHYDRATES, ETC.	STRAINS																
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	F1	F2	F3	T1	T2	T3
Glucose	+	++++	+++	+	++++	++++	++++	+	+	+	++++	++++	++++	++++	++++	++++	++++
Levulose	+	++++	±	++++	++++	++++	+	+	+	++++	++++	++++	++++	++++	++++	++++	++++
Maltose	+	++++	±	+	++	++	+	++	+	++++	++++	++++	++++	++++	++++	++++	++++
Lactose	±	−	−	−	−	−	−	−	±?	−	−	−	−	−	++++	−	++++
Sucrose	+	±	±?	−	−	−	−	−	−	−	++++	++++	±	±	+	++++	++++
Mannose	+	++++	−	−	++++	++++	++++	±	±	±	++++	+	+	+++	++++	−	++++
Melezitose	−	++++	−	−	+++	+++	±	−	−	−	−	−	−	++++	++++	−	+++
Raffinose	±?	−	−	−	−	−	−	−	±?	±	++++	±	++++	−	±?	−	±?
Dextrin	+	−	−	−	−	−	−	−	±	−	++++	±	+	±	±?	−	++++
Arabinose	−	−	++	++++	−	−	−	++++	−	++++	−	−	−	−	+	−	++
Xylose	−	−	±?	−	−	−	−	++++	−	++++	−	−	−	−	±	−	++
Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++++	−	+++
Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++++	−	±?
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Mannitol	±?	+++	−	−	±?	±?	±	±?	−	−	−	±	±	++++	++++	−	±
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+++
Inosite	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	−
Starch	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Glycerol	−	±?	−	−	−	−	−	−	−	−	−	−	−	−	+	−	±?
Salicin	±	++	−	−	++	++	+	++	±?	−	++++	−	−	++	++++	−	++++
Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Milk	+	−	−	−	+	+	−	+	+	−	−	−	−	−	+	−	+

while neither A2 nor F3 does. On the other hand, F3 ferments dulcitol, while A2 and A9 do not.

A4 and A8 resembled each other at the time of the isolation of A8. Similarly A2 resembled A7 at the time A7 was isolated. While A2 and A7 still resemble each other after being carried as stock cultures for two years, A4 and A8 have lost much of their similarity. A8 ferments four carbohydrates which are not acted upon by A4. The former also acts upon milk, while the latter does not.

Strain 11 resembles A11 in every way save degree of action.

Strain 13 resembles A1 except that A1 ferments mannitol and milk. Strains 87, 85, 29 and 24 are the same in their reactions as 13. Strains 34 and 71 were lost.

There is a general similarity in the fermentation reactions of the newly isolated strains 11, 12, 13, 15, 23, 24, 28, 29, 32, 72, 73, 85 and 87. The carbohydrates which are fermented are limited to glucose, levulose, maltose, lactose, sucrose, mannose, raffinose,

TABLE 2

ng fermentation reactions

		STRAINS																				
T1	T	T3	K1	K2	RH	Alf	11	12	13	15	23	24	28	29	32	34	71	72	73	85	87
++++		++++	++	+++	++	++++	++++	++++	++++	++++	++++	++	+	++++	+	−	−	+	++++	+++	++++
++++		++++	+	−	+	+	++	*	++	++	*	+	+	+	+	−	−	+	+	+	+
++++		++++	++	−	++	++++	++++	++++	++++	+++	+	+	+	++++	+	−	−	++++	++++	++++	++++
++++		++++	++	+++	++	*	−	−	*	−	*	*	−	+	−	−	−	−	−	+	*
+		++++	+	−	*?	−	+	−	+	*	*	*	*	+	*	−	−	*?	+	+	*
++++		++++	++	+++	+++	*	++++	*	++	+	*	+	+	+	*	−	−	*?	++++	+	+
++++		+++	−	−	*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
*?		*?	+	−	++	−	++++	−	++++	*	−	+	*	+	*	−	−	−	+	+	+++
*?		++++	−	−	*?	−	++++	−	++++	*	−	+	+	++++	+	−	−	−	+++	++++	++++
+		++	−	−	*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
*		++	−	−	*?	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
++++		+++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
++++		*?	−	−	−	−	−	−	−	−	−	−	−	−	*?	−	−	−	−	−	−
−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
++++		*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−		+++	−	−	*?	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
++		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+		*?	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
++++		++++	−	−	+	−	+++	−	+	++	−	*	−	+	−	−	−	−	+	+	+
−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
+		+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

dextrin and salicin. Arabinose, xylose, mannitol and milk were acted upon by many of the cultures carried in the stock medium, but they were not utilized, on the other hand, by the recently isolated strains.

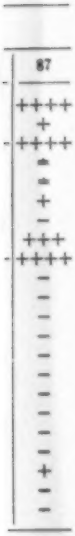
The newly isolated strains described here fall into three groups: Type I includes 11, 15, 28 and 73. This group resembles A11 (*L. plantari*) of the type cultures. Type II includes 12, 23 and 72, which resemble culture Alf, but none of the type cultures. Type III includes 13, 24, 29, 85 and 87 and resembles A1 (*L. leichmanni*) of the type cultures.

BOOK B

TIGHT

BOUND

HTLY



Strains T1, T2 and T3 (teeth strains), K1 (*L. acidophilus*), K2 (*L. bulgaricus*), and RH (*L. acidophilus*) have one reaction in common which is not shared by most of the organisms isolated from grain and soil. The organisms of dental origin, those isolated from milk, and those from feces of animals on a high carbohydrate diet ferment lactose very readily. On the other hand, the soil organisms either do not ferment lactose at all or exert only a slight action.

SEROLOGICAL RELATIONSHIPS

Representative strains of type cultures and newly isolated organisms were selected for serological study. The antigens were prepared as follows: Twenty-four-hour old cultures of the organisms in yeast-water broth containing peptone and glucose were centrifuged, the supernatant fluid removed and the bacterial cells washed and centrifuged 3 times in sterile physiological saline solution. The residue was finally made up to a turbidity of 8 to 10 on the McFarland nephelometer scale with sterile saline, and a loopful of the final suspension plated out as a check on its purity. Rabbits were immunized with the unheated, washed antigens by repeated injections in the marginal ear vein until the agglutination titer of the serum against the homologous organism was at least 1:800.

The following eight cultures were used to prepare the immune sera which had the indicated agglutination titers:

A1	1:800
A5	1:1600
A9	1:1600
A10	1:800
Alf	1:1600
11	1:1600
72	1:1600
RH	1:800

AGGLUTINATION REACTIONS

The agglutination reactions of these sera against the strains of lactobacilli studied are shown in table 3. They were determined with washed antigens having a turbidity of 1:1.5. Four dilutions

TABLE 3

Showing agglutination reactions

SERA	ANTIGENIC STRAINS																																		
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	F1	F2	F3	T1	T2	T3	K1	K2	RH	Alf	11	12	13	15	23	24	28	29	32	34	71	72	73	85	87
A1	++	–	–	–	–	–	?		?	?	–	–	–	?	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–
A5	–	–	–	–	++	++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–
A9	–	–	–	–	–	–	?	–	++	?	–	–	–	?	–	–	–	–	–	–	–	++	++	++	++	–	–	–	–	–	–	–	–	–	–	–
A10	–	++	–	–	–	–	++	–	–	++	–	–	–	++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alf	–	–	–	–	–	–	?	–	–	?	–	–	–	?	–	–	–	–	–	–	++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++	–	–	–	–	–	–	–	–	–	–	–
72	+	–	–	–	–	–	–	–	–	–	–	+	?	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	–	–
RH	–	–	–	–	–	–	?	–	–	?	–	–	–	?	–	–	–	–	–	++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

TABLE 4

Giving complement fixation reactions

SERA	ANTIGENIC STRAINS																																		
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	F1	F2	F3	T1	T2	T3	K1	K2	RH	Alf	11	12	13	15	23	24	28	29	32	34	71	72	73	85	87
A5	–	–	–	–	++++	++++	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Alf	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–
72	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	–	–

of serum were used, namely 1:100, 1:200 1:400 and 1:800, and a control containing no serum. The readings were made after two hours incubation at 56°C. and again after remaining over-night at 3 to 4°C. The key to the symbols used in the table of agglutination reactions is as follows:

− = no agglutination
? = some agglutination in control tube
+ = agglutination in 1:100 or 1:200
++ = agglutination in 1:400 or 1:800

Most of the sera showed a marked proagglutination phase in the dilutions lower than 1:100, but all sera agglutinated the homologous organism at dilutions of 1:100 or above.

Several points should be noted in the table of agglutination reactions. Although A1, F1 and F2 are all type cultures of *L. leichmanni*, neither F1 nor F2 is agglutinated by A1 antiserum. This agrees as far as it goes with the interpretation of the results of the fermentation reactions. Strain A1 is unlike F1 and F2. Unfortunately no antiserum was prepared for either F1 or F2, and their serological similarity remains undetermined. It may also be significant that an agglutination reaction was obtained in the lower dilutions with strains 29 and 85. The last two organisms were found to be similar in their fermentation reactions to A1.

A5 and A6 are serologically the same. Although an agglutination was obtained in the lower dilutions with A5 antiserum and antigens of cultures of Alf and 24, neither of these cultures resembles A5 or A6 in its fermentation reactions. Strain 24 resembles A1 by the fermentation tests, while Alf falls into another group. There is no agglutination of either 24 or A1 by the antiserum of Alf.

A9 antiserum does not agglutinate A2 or F3 antigens. This agrees with the findings of the fermentation reactions. Although no two of these three strains show identical fermentation reactions, A2 more closely resembles F3 than A9 does either A2 or F3. Neither F3 nor A2 antiserum is available to determine whether A2 and F3 antigens and antisera cross agglutinate.

A9 antiserum also agglutinated 11, 12, 13 and 15 antigens even

in the higher dilutions. Inasmuch as No. 11 antiserum also agglutinates 11, 12, 13 and 15 antigens in the same high dilutions, it might be concluded that these cultures are serologically the same. Notwithstanding this fact, antiserum 11 does not agglutinate A9 antigen, and the fermentation reactions of these strains are quite different.

There is no serological similarity between cultures 11, 28, 73 and A11 as a group. Neither is there between Alf, 23, and 72, or between 13, 24, 29, 85, and 87. These groups have similar fermentation reactions within themselves. Antiserum RH does not agglutinate antigen K1, though both are *L. acidophilus*.

Strains 71, 72 and 73 are serologically similar, although 72 and 73 do not have similar fermentation reactions. While antiserum 72 agglutinates antigens A1 and F1 in the lower dilutions, and causes possibly a slight reaction with F2 antigen, there is no marked similarity in their fermentative properties. F1 more closely resembles 72 than does A1. However, A1, F1 and F2 are all *L. leichmanni* type cultures.

It is perhaps significant that antiserum A10 agglutinates antigens A2, A7 and F3 in dilutions as high as it agglutinates its specific antigen. Strain A7 was, as has been previously mentioned, similar in its fermentation reactions to A2 when first isolated. A2 and F3 are both *L. delbrücki*. While A9 is likewise an *L. delbrücki* type culture, it is not agglutinated. It has already been noted that A2 and F3 more nearly resemble each other in their fermentation reactions than either one resembles A9. However, A10 ferments the pentoses easily, while A2, A7 and F3 do not.

COMPLEMENT FIXATION REACTIONS

Kulp and Rettger (1924) failed to obtain satisfactory agglutination reactions with their strains of *L. acidophilus* and *L.bulgaricus* because of spontaneous agglutination. They finally resorted to the complement fixation test. Morishita (1928), in working with organisms isolated from dental caries, found the agglutination method satisfactory after *certain preliminary treatment* of the suspended antigen. In the present work only three strains

agglutinated spontaneously, and then not sufficiently to mask true agglutination. However, for the sake of comparison, complement fixation tests were made with four immune sera against the 36 antigens employed in the agglutination experiments.

The sera used were four of those which were employed in the agglutination work, namely A5, Alf, 11 and 72. They were run in two dilutions and well beyond the anticomplementary range. The antigens were prepared from the stock suspensions of washed viable organisms in sterile saline solution. The degrees of fixation are indicated in table 4 on the −, +, ++, +++, and ++++ basis.

The complement fixation reactions obtained here agree only in part with the agglutination reactions. Whenever a 4+ complement fixation was obtained, a 2+ agglutination reaction was observed. However, this is as far as the correlation between the two systems went. The 1+ complement fixations do not in any case agree with agglutinations reactions obtained in these instances.

While the complement fixation technique is a very delicate one, and to a certain extent confirms the agglutination reaction, it is also very cumbersome. The agglutination method may be used successfully with the strains of organisms used here. Strains that are naturally subject to self-agglutination may by certain treatment[5] be made to serve as satisfactory agglutination antigens.

DISCUSSION

Several points may be emphasized here. It is often very difficult to establish the identity of any one isolated strain of Lactobacillus definitely. At times there are greater differences between the fermentation reactions of two given strains of the same organism than are seen between two individuals of different species. Henneberg (1904) divided his *L. leichmanni* strains into three groups. *L. leichmanni* I did not ferment lactose, raffinose, dextrin or inulin, but attacked alpha-methyl glucoside.

[5] Rapid and repeated transfer of cultures (three to four hours) and centrifugation of the washed cells to remove the coarser clumps.

L. leichmanni II fermented all of these, while *L. leichmanni* III acted upon all but raffinose and the glucoside. Except for the amount of acid produced, *L. listeri* corresponded exactly with *L. leichmanni* II, save possibly in the instance of rhamnose; no data are given on the action of *L. leichmanni* II upon this sugar.

Of the fifteen organisms listed by Henneberg there are only four which ferment lactose strongly, eight which do not attack it at all, and two which act upon this sugar only to a limited extent. The lactose fermentation of one organism is not listed.

The evidence offered in this paper by the fermentation and agglutination reactions of known strains of lactobacilli is only in partial agreement with our knowledge of the sources of these strains. In some instances the fermentation reactions are confirmed by the agglutination reactions, while in others they are not. The agreements almost balance the disagreements. The complement fixation reactions only in part support the findings of the agglutination tests. The morphology of the organisms and colony formation merely serve as a preliminary means of identification and cannot be used by themselves as a means of distinguishing the individual members of the genus employed in this investigation.

Work with known members of this group is a discouraging preliminary to work with unknown members. If one cannot find agreement with known strains (sometimes subcultures of the same original strains from two different laboratories do not agree) then it is useless to place too much emphasis upon the fermentation or agglutination reactions. While there can be no question regarding the delicacy of the serological and fermentation methods, the significance of these sensitive reactions may perhaps be overemphasized in the instance of the lactobacilli when one considers the variability which some of these organisms at times show, at least in artificial culture.

The tendency of some writers, in describing a given organism of the Lactobacillus group, to conclude that it is in certain respects like *L. delbrücki*, or that it "resembles" *L. acidophilus*, is perhaps fortunate, and indeed judicious, particularly when their observations are based on incomplete data. To label an or-

ganism definitely as *L. acidophilus* or *L. delbrücki* simply because it bears certain general resemblances cannot be too strongly condemned.

There is one characteristic which most of the organisms isolated from soil, grains and fruits lack, but which is possessed by *L. acidophilus* and *L. bulgaricus* as described by Kulp and Rettger (1924), and by *L. odontolyticus* as described by Morishita (1929). The soil organisms employed here do not as a rule ferment lactose, while *L. bulgaricus*, *L. acidophilus* and *L. odontolyticus* utilize this carbohydrate readily. One is not justified, however, in arbitrarily taking one or two properties of an organism to the exclusion of all others, and concluding that all organisms possessing this property are the same. Rather, the writers wish to point out the dissimilarities between this large group of lactobacilli isolated from soil, and the more specific types of *L. acidophilus*, *L. bulgaricus* and *L. odontolyticus*.

Until further and more substantial data are available it is perhaps unwise to attempt further distinctions. However, it has been noted that the organisms isolated by the writers fall into several groups on the basis of their fermentation characteristics. Type I includes strains 11, 15, 28 and F3 which might be called *L. plantari*. Type II includes strains 12, 23, F2 and Alf, which act upon relatively few of the carbohydrates employed in these tests. Type III is the largest group including 13, 24, 29, 85 and 87 and might be designated as *L. leichmanni*. Type IV was not often met with, but like *L. pentoaceticus*, *L. fermentatae* and *L. lycopercici*, these organisms fermented one or more pentoses readily. A pentose fermenter was encountered and isolated in four instances, but A8 was the only strain available for these studies.

The ability of some organims to form gas from carbohydrates has been mentioned by some writers. None of the strains described in this paper possess this characteristic now. Even *L. lycopercici*, which produced large amounts of gas from tomato juice when first received in this laboratory, has lost this property. Hence it could not be used as a means of differentiation.

The question of source of the soil lactobacilli is an interesting

one. It was observed in this work that when a sample of grain was protected from animal contamination it ceased after a while to yield lactobacilli. This was first noted by the writers when they originally attempted to isolate what was then known as *B. volutans*. All efforts to isolate aciduric organisms from samples of corn and corn flour which had been in sealed jars and bottles in the laboratory for several months met with failure. When fresh samples were obtained from a feed store isolation was effected without difficulty.

Although it was surmised that samples from feed-stores and cereal mills were continually being infected by rodents, several attempts to isolate the organism from the intestinal contents of mice trapped in the store-room were not successful. It is possible, nevertheless, that the fecal material of certain animals is the ultimate source of these organisms. This speculation is strengthened when one considers the prevalence of the lactobacilli in surface soil of many varieties all of which were subject to fecal contamination.

SUMMARY

Thirty-six strains of Lactobacilli representing strains isolated from soil, grain, fecal material, milk products and dental caries are described. The media used and the methods of isolation are detailed.

The fermentation reactions of the organisms upon 20 carbohydrates and related substances, their action in milk, broth and gelatin, and their acid production at four incubation temperatures, are listed.

Agglutination reactions and complement fixation tests of the 36 strains with antisera of representative strains are also listed and results compared with fermentation reactions.

Lactobacilli isolated from soil and grain did not as a rule ferment lactose. This marks them as a group distinct from *L. acidophilus*, *L. bulgaricus* and *L. odontolyticus*.

Of the lactose non-fermenters, four types are more or less definitely indicated on the basis of their fermentation reactions.

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THE INFLUENCE OF H-ION CONCENTRATION ON THE SPORULATION OF B. WELCHII

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It is well known that *B. Welchii* does not sporulate readily. When grown in ordinary culture media scanty spore production is usually noted even after prolonged incubation. This applies particularly to media containing a fermentable carbohydrate even though in very small amount. The literature contains few studies on the environmental factor or factors which determine sporulation on the part of this organism. Such reports as have been made would suggest that the reaction of the substrate exercises the controlling influence. Grassberger and Schottenfroth in 1900 reported that the sporulation activity of their anaerobic immobile butyric acid bacillus was increased by alkalinity in the medium and that the optimal degree of alkalinity varied with different strains. Some of these strains may not have been *B. Welchii* as the species was not adequately identified at that time. Noguchi in 1907 employed a sugar-free bouillon with an alkalinity ranging from N/50 to N/75 NaOH as a basic medium and added various carbohydrates in 1 per cent amount. He found that if acidity was produced by the growth of *B. Welchii* no spores were formed. Fitzgerald in 1911, working with Noguchi and using his methods, confirmed in general his findings. She concluded that the optimal alkalinity zone for spore formation ranged from N/200 to N/50 NaOH, that the presence of NaCl in the medium, ranging from 0.5 to 5 per cent, was not a factor influencing spore formation but that the presence of certain unfermented carbohydrates in the basal medium, especially mannitol and amylodin, enhanced spore formation while even in the presence of acid formed from

raffinose, spore formation was increased. Simonds (1915) in his extensive monograph on *B. Welchii* states that "spore formation is inconstant and occurs only in alkaline media, never in pure cultures containing a fermentable sugar or a free acid." In some *in vitro* experiments with fecal suspensions he found that sterilized preparations seeded with *B. Welchii* did not show spores after incubation if the acidity was made equal to 1.0 per cent normal hydrochloric or acetic acid with phenolphthalein as an indicator. Also when a sugar, such as lactose, was added in 1 per cent amount to unsterilized fecal suspensions containing *B. Welchii*, no spores were observed after incubation if the final degree of acidity was greater than 4.0 per cent normal. Nye (1927) in connection with a study of *B. Welchii* spore formation in the intestines of cases exhibiting achylia gastrica states that a 1.0 per cent fecal suspension adjusted to 1.0 and 4.0 per cent acid (normal HCl), using phenolphthalein as an indicator, has a pH of approximately 7.5 and 5.0 respectively. He demonstrated in this study, that individuals with achylia gastrica, with or without anemia generally show very high counts for *B. Welchii* spores in stool specimens and he ascribes this phenomenon to the probable greater degree of alkalinity of the content of the small intestine of such cases.

The purpose of the present investigation has been to determine just what degree of H-ion concentration in an appropriate medium is most conducive to the sporulation of *B. Welchii*. To this study there are also appended observations on a series of gastric anacidity cases as regards the correlation of gastric and duodenal content reaction and the *B. Welchii* spore counts for the feces. In the *in vitro* tests the aim has been to employ a medium the reaction of which would show minimal changes as a result of the growth activities of this bacillus.

EXPERIMENT I

Medium. The basal medium employed throughout these experiments consisted of a sugar-free, well-buffered nutrient broth. It was prepared as follows: To 2 liters of distilled water, 2 pounds of minced fresh beef heart were added and boiled over

the free flame with frequent stirring for 15 minutes. It was then passed through canton flannel and absorbent cotton, any loss in the filtrate being adjusted with distilled water, and then autoclaved for 20 minutes at 15 pounds pressure. This filtrate was next inoculated with *B. coli* and incubated at 37°C. for 24 hours. A test was made for sugar using Benedict's method and, if found sugar-free, the broth was cleared with egg white and passed through filter paper and to the filtrate was added 1 per cent peptone (Difco). After the latter had been brought into solution by moderate heat, the reaction was adjusted to pH 7.0 and further clearing effected by autoclaving and filtering through paper. The filtrate was then distributed in flasks in 250 cc. amounts and these lots were severally adjusted with 10 per cent solutions of Na_2CO_3 and HCl, using Clark and Lubs indicators, to the H-ion concentrations detailed in the several tables. To each flask of adjusted medium employed in the experiment designated in table 1 as (*a*), 0.5 per cent potassium diphosphate was added as a buffer. In the other tests (*b*, *c* and *d*) changes in buffering were made as indicated below. Following autoclaving for 15 minutes at 20 pounds, the flasks were re-titrated, reaction adjustments made, where necessary, and the content of each tubed in about 15 cc. amounts in 16 mm. diameter tubes. Prior to filling, a small piece of sterile calcium carbonate had been placed in each tube to stabilize further the reaction during the growth period. The tubes were finally autoclaved and seeded as described below. The *B. Welchii* strain employed in the experiments detailed in table 1 had been isolated from a pernicious anemia case several years ago, was entirely typical culturally and had been purified by single cell culture. Six tubes at each reaction were seeded. The H-ion concentrations before inoculation were determined then and at the indicated time periods following seeding and incubation. At each of the periods films for staining were also prepared from a sample tube at each reaction and the tube was then discarded. The Kopeloff and Beerman modification of the gram method was employed. The percentage of spores, both free and intracellular was then determined per 100 organisms at each reaction using a net ocular micrometer. A

sufficient number were counted to obtain a fair average. Table 1 records the results of four series of tests employing six to ten reactions in each of the first three. They were as follows:

(*a*) Medium as described above at primary reactions 6.2, 6.6, 6.8, 7.0, 7.3, 7.5, 7.7, 7.8, 8.0 and 8.2. Seedings were made by adding a few drops of a 48-hour culture of *B. Welchii* in cooked meat medium. There are practically no bacilli in the spore stage in such a culture, but even if there were and they did not develop into rods, dilution would be so great as to make their presence negligible.

(*b*) Reactions 6.6, 6.8, 7.0, 7.3, 7.5, 7.7, 8.1, 8.4 and 9.0. The technic followed was the same as for (a) except that the amount of potassium diphosphate in the medium was increased to 1.0 per cent in an attempt to keep the reaction more constant. Also, seedings were made from a 48-hour culture in plain nutrient broth. As the 8.4 and 9.0 reactions did not remain constant after autoclaving, 0.5 per cent acid sodium phosphate was substituted for the 1.0 per cent potassium diphosphate. The reactions remained constant on autoclaving but no growth of *B. Welchii* occurred.

(*c*) Reactions 6.2, 6.5, 6.8, 7.0, 7.2 and 7.5. In this series the technic was the same as for series (a) except that 0.5 per cent acid sodium phosphate was used as a buffer instead of potassium diphosphate. At several of the reactions the growth was not visible for several days but due allowance was made for this delay in carrying out the observations.

(*d*) A small supplementary series was run at primary reactions 7.0 and 7.2 in which the conditions were the same as for series c.

RESULTS

In series (a) the degree of growth as indicated by turbidity was about the same for reactions between 6.2 and 7.8; at 8.0 and 8.2 it was scantier and under the above described experimental conditions none was obtained at 8.5 and more alkaline reactions. The medium was not entirely free from fermentable carbohydrate as shown by the early appearance of some gas. There was, however, only a minute amount present as the increase in acidity

TABLE 1

Sporulation of B. Welchii in fluid medium of various reactions

pH	MEDIUM	1 DAY		2 DAYS		5 DAYS		7 DAYS		9 DAYS		12 DAYS	
		pH	Spores	pH	Spores	pH	Spores	pH	Spores	pH	Spores	pH	Spores
			per cent		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*
6.2	a	6.1	0	6.2	0	6.1	0	6.1	0	6.1	15	6.1	10
	c	6.1	0	6.1	0	6.1	0	6.2	0	6.2	5	6.2	2
6.5	c	6.4	0	6.5	0	6.5	2½	6.5	0	6.5	5	6.5	2
6.6	a	6.1	0	6.3	0	6.3	0	6.3	0	6.3	0	6.3	8
	b	6.5	2	6.5	2.2	6.6	6	6.6	10	6.6	9	6.6	11
6.8	a	6.8	12	6.8	1	6.8	25	6.8	31				
	b	6.6	23	6.8	10	6.8	20	6.8	23	6.8	16	6.8	10
	c	6.7	9	6.7	6	6.7	2	6.8	7	6.7	7	6.9	5
7.0	a	6.8	6	6.8	4			6.8	28				
	b	7.0	46	7.1	50	7.0	30	7.0	16	7.0	25	7.0	15
	c	6.9	5	6.9	10	6.8	6	6.7	6	6.7	7	6.7	5
	d	6.7	9	6.7	8	6.8	11	6.9	8	6.8	18	6.8	8
7.2	c	6.8	6	6.8	4	6.8	5	6.8	5	6.8	8	6.8	4
	d	7.0	2	7.0	21	6.9	12	7.0	18	7.0	17	7.0	32
7.3	a	7.3	1	7.3	1	7.1	21	7.1	29				
	b	7.5	13	7.5	32	7.5	53	7.5	31	7.5	9	7.5	30
7.5	a	7.1	80	7.1	40	7.1	38	7.1	61	7.1	69	7.1	73
	b	7.3	49	7.4	46	7.4	57	7.4	41	7.4	33	7.4	28
	c	7.5	3	7.5	22	7.4	51	7.5	34	7.5	20	7.5	21
7.7	a	7.3	11	7.2	20	7.2	49	7.2	85				
	b	7.7	23	7.7	45	7.7	58	7.7	48	7.7	11	7.7	37
7.8	a	7.3	55	7.3	82	7.3	43	7.3	68	7.3	72	7.3	86
8.0	a	7.8	44	7.7	33	7.7	18	7.7	52	7.7	76	7.7	75
8.1	b	8.0	1	8.0	2½	8.0	2½	7.8	35	7.9	25	7.8	30
8.2	a	7.8	55	7.7	72	7.7	35	7.7	54	7.7	64	7.7	50

was very slight at most reactions. The medium with acid sodium phosphate held nearer to the initial reactions than did that with potassium diphosphate but on the other hand growth in the latter was rather less vigorous.

As is shown in table 1 the reactions were held quite constant throughout the period of the test except in a few instances in series (a). The increase in acidity as the result of growth in series (a) ranged from 0.1 to 0.5 points whereas in (b) and (c) there was no increase or none greater than 0.2 points except in one instance.

Our findings demonstrate clearly that, given a suitable reaction in the menstruum, *B. Welchii* will sporulate freely and that this spore-inducing reaction lies between pH 6.8 and the limit of growth on the alkaline side, which was with this particular medium 8.2. The maximal spore production zone lies on the alkaline side of absolute neutrality starting at about pH 7.1. At reactions from pH 6.1 to 6.5 inclusive practically no spores were noted until 9 days incubation had elapsed and then in a percentage of no more than 2 to 15. At an initial reaction of 6.6 spores begin to be in evidence within 24 hours but at 6.8 larger numbers were observed and about 2 per cent were intracellular. Judging from the number of organisms seen in films this reaction seemed to favor optimal growth. Beginning with 7.1 and more alkaline reactions spore formation occurred promptly and freely, with about the same percentage (from about 10 to 80) generally observed after 24 hours incubation as after 9 to 12 days. Intracellular spores never amounted to more than 5 per cent of the total organism count and were not noted at all after 48 hours. In this optimal zone the active period of spore formation was thus apparently within the first 48 hours of growth.

EXPERIMENT II

As the above tests were carried out with a strain of *B. Welchii* which had been under cultivation for several years it was obviously desirable to substantiate the findings by further tests with one recently isolated.[1] The medium and methods were the

[1] We are indebted to Miss Madeline Henriques for assistance in this part of the study.

TABLE 2

Sporulation of a recently isolated strain of B. Welchii

MEDIUM	ORIGINAL pH	1ST DAY			2ND DAY			5TH DAY			7TH DAY		
		pH	Spores	Remarks	pH	Spores	Remarks	pH	Spores	Remarks	pH	Spores	Remarks
			per cent			*per cent*			*per cent*			*per cent*	
1	6.2	6.2	0	Single normal rods	6.2	0	Single normal rods	6.4	0	Single normal rods	6.4	0	Single normal rods
2	6.5	6.3	0	Single normal rods	6.3	0	Single normal rods	6.5	0	Single normal rods	6.5	5	Single normal rods
3	6.7	6.4	4	Single normal rods	6.4	0	Single normal rods	6.6	0	Single normal rods	6.5	3	Single normal rods
4	7.0	6.8	7	Single normal rods	6.8	2	Single normal rods	6.8	3	Single normal rods	6.8	5	Single normal rods
5	7.4	7.0	3	Single normal rods	7.0	1	Single normal rods	6.8	0	Rods tend to chain	7.0	5	Organisms tend to chain
6	7.6	7.0	12	Single normal rods	7.3	0	Rods tend to chain	7.0	3	Rods tend to chain	7.0	7	Organisms fairly scarce. Tend to chain and are thinner
7	7.9	7.3	32	Single normal rods	7.7	19	Rods tend to chain	7.4	35	Rods tend to chain	7.4	29	Organisms chain and are thin
8	8.2	7.8	1	Single normal rods	7.7	32	Rods tend to chain	7.6	19	Rods tend to chain, thinner	7.5	44	Organisms tend to chain and thin

same as that used in experiment Ia, the only difference being that the *B. Welchii* strain had been isolated from the intestine of a pernicious anemia case a few weeks before and the tubes were seeded from the third generation of growth. As is shown in table 2, the results were much the same as for experiment I, the zone of early spore formation ranging from reaction 6.7 to 8.2 with an optimum zone between 7.6 and 7.9. After 24 hours there were many more rods with spores than free spores at most reactions but in 48 hours and later practically all the spores were in the free state. The zone of prolific spore formation with this strain ranged from primary reactions of 7.9 to 8.2 which was somewhat more alkaline than with the first and older strain employed. With both, however, there was a complete inhibition of spore production during the first 5 days at 6.5 and more acid reactions and a zone of spore production starting at about 6.7. With this strain also the organisms tended to grow in chains in the medium with a primary alkaline reaction. This was noted after 2 days incubation in the tubes at pH 7.6 and more alkaline reactions and after 5 days in the tubes of a primary pH of 7.4 and over, although the reaction in the former was pH 7.0 at that observation period. The thinner appearance and relative scarcity of the rods in the tubes at pH 7.6 to 8.2 after 7 days suggest that a constant alkaline reaction is unfavorable for this organism.

EXPERIMENT III

Fitzgerald (1911) claimed that spore formation by *B. Welchii* could be induced by the addition to peptone broth of such carbon compounds as arabinose, raffinose, inulin, mannitol, dulcitol and amydolin, with mannitol and amydolin giving the best results. The strain employed did not ferment mannitol or any of the other carbohydrates except raffinose. With raffinose, however, in spite of the presence of acid due to its fermentation, numerous spores were noted. We have not been able to confirm clearly these findings as far as mannitol and raffinose are concerned when they were added to our medium, as is indicated in table 3. Sterile solutions of these carbohydrates were added to tubes of our basal

TABLE 3

Sporulation of B. Welchii in broth medium containing mannitol and raffinose

MEDIUM	ORIGINAL pH	1 day			2 days			7 days		
		pH	Spores	Remarks	pH	Spores	Remarks	pH	Spores	Remarks
			per cent			*per cent*			*per cent*	
		MANNITOL								
1	6.2	6.1	0	Single normal rods	6.3	0	Single normal rods	6.3	5	Single normal rods
2	6.5	6.4	0	Single normal rods	6.4	0	Single normal rods	6.4	6	Rods tend to chain
3	6.7	6.5	0	Single normal rods	6.5	0	Single normal rods	6.5	6	Rods tend to chain
4	7.4	7.0	0	Single normal rods	7.0	0	Single normal rods	6.9	5	Rods tend to chain
5	7.9	7.2	5	Single normal rods	7.4	5	Single normal rods	7.3	46	Rods tend to chain
		RAFFINOSE								
1	6.2	6.1	0	Single normal rods	6.1	0	Single normal rods	6.0	0	Rods thinner and tend to chain
2	6.5	6.2	0	Single normal rods	6.1	0	Single normal rods	6.0	0	Rods thinner and tend to chain
3	6.7	6.3	0	Single normal rods	6.2	0	Single normal rods	6.0	0	Rods thinner and tend to chain
4	7.4	6.5	0	Single normal rods	6.3	0	Single normal rods	6.0	2	Rods thinner and tend to chain
5	7.9	6.9	1	Single normal rods	6.5	8	Rods tend to chain	6.2	23	Rods thinner and tend to chain

medium in amount sufficient to make a final 1 per cent strength in the reactions designated in the tabulation. Mannitol evidently was not split by this strain which was the recently isolated one of experiment II and there was no clear-cut evidence that its presence induced spore formation except that 5 per cent of spores were found in the pH 6.2 tube whereas there were none in the sugar-free lot (table 2). Raffinose was split slowly with the accumulation of a moderate amount of acid (a reaction of 6.0 to 6.2 in all tubes after 7 days) which was sufficient to inhibit spore formation except in the tube with a primary 7.9 reaction where 23 per cent of spores were found after 7 days in spite of the fact that the reaction had then reached pH 6.2. There was thus no evidence that these two carbohydrates favored spore formation in more than very slight degree.

In summarizing the results of these experiments it may be said that the reaction of the medium was found of decisive influence on spore formation by *B. Welchii*. Acidity was inhibitory and in concentration greater than pH 6.6 prevented any spore formation for 7 days. With longer incubation a small percentage of spores were noted in the zone between 6.0 and 6.6. With both strains, early sporulation started rather abruptly at 6.8 and continued through increasing alkalinity to the limiting reaction for growth, with the maximum numbers occurring between 7.5 and 8.2. In this zone decreased vigor of growth was noted which suggests that unfavorable environment conditions were correlated with sporulation. No evidence appeared that the presence of mannitol or raffinose in the culture medium induced spore formation as was claimed by Noguchi and also Fitzgerald.

REACTION WITHIN THE HUMAN INTESTINE AND SPORULATION OF B. WELCHII

Years ago Herter (1906–7) called attention to the fact that *B. Welchii* spores are unusually abundant in fecal material from pernicious anemia patients. This finding has been thoroughly confirmed within recent years by Simonds (1915), Kahn (1924), Moench, Kahn and Torrey (1925), Nye (1927), Davidson (1927) (1928)). Nye (1927), employing a cultural technic which per-

mitted accurate comparative spore counts, found an average of 46,000,000 *B. Welchii* spores per gram of feces from 11 cases of pernicious anemia as contrasted with 240 per gram for 6 normal individuals, or approximately 200,000 times as many in the former as the latter. Davidson (1928) estimated that the excess of pernicious anemia counts over that of normals amounted to 1000 to 10,000 times. Nye (1927) further found that in five cases of achylia gastrica with secondary anemia the average *B. Welchii* spore count was even higher than with his series of pernicious anemia cases, viz., 51,000,000 per gram of feces. As the pernicious anemia cases showed the typical complete absence of gastric acidity and in view of the fact that under cultural conditions alkalinity seems to encourage spore formation by *B. Welchii*, he suggested that the high counts were due to a favorable alkaline reaction of the small intestine content. Knott (1927) in 27 normals found that the reaction of the resting content of the duodenum averaged 7.0 with a range of pH 6.6 to 7.2. Van der Reis (1925) has reported reactions for various levels of the small intestine in a series of 63 normals during the resting period, finding a range of 5.9 to 6.6 for the upper part, 6.2 to 6.7 for the middle and 6.2 to 7.3 for the lower section, but he does not state definitely how many hours after a meal these determinations were made. The findings of McClendon, Bissell, Lowe and Meyer (1920) for the reactions of the mid-portion of the small intestine content showed, 1½ to 3 hours after a meal, for the middle part of the jejunum an average of pH 5.2 with a maximal acidity of pH 4.1 and a minimal of 6.5. These observations were made over a period of 4 days. Long and Fenger (1917) had previously shown that under somewhat similar conditions, in 3 normal human subjects 6½ hours after an ordinary meal, the material beyond the duodenal-jejunal juncture gave a reaction of pH 5.83. Very recently Paulson (1929) has reported that in three cases upon whom ileostomies had been instituted for the relief of ulcerative colitis the H-ion concentration of the lower ileum content was, with one exception in 43 determinations, alkaline regardless of the type or amount of food ingested. For case 1 the average reaction was pH 7.65, for case 2 pH 7.47 and for case 3 pH 7.4.

Although gram-positive rods were noted, *B. Welchii* was not recovered or its spores noted, although its presence was occasionally indicated culturally.

The above observations, considered in connection with our finding that a reaction in the substrate more acid than pH 6.6 almost completely inhibits *B. Welchii* spore formation, indicate, as Nye (1927) has suggested, that the periodic waves of acid gastric secretion passing along the small intestine prevent any *B. Welchii* vegetating there from sporulating except perhaps occasionally in the terminal part of the ileum. With gastric anacidity, however, the reaction of the small intestine content is necessarily to some degree alkaline. One of us (T) has found in a series of 21 cases of gastric anacidity that the average reaction of the duodenal (mid-portion) content on a fasting stomach (specimens taken prior to breakfast) was pH 7.35 with a range from pH 6.8 to 7.9. These determinations were made colorimetrically with the Clark-Lubs indicators within 1 to 3 hours after the specimens had been obtained with a Rehfuss or Lyon drainage tube.[2] No precautions were taken to prevent loss of CO_2. Knott (1927) has reported practically the same reactions for the resting duodenal contents in 15 cases of gastric achlorhydria, viz., an average of pH 7.4 with a range from pH 7.0 to 7.8. Apparently no determinations have been reported for the middle and lower levels of the small human intestine in achlorhydria cases, but it is reasonable to suppose that the reactions would be definitely alkaline and perhaps to a greater degree than for the duodenum. Taken in connection with our determinations, the indications are, then, that the reaction of the small intestine in cases of achlorhydria gastrica would be favorable for spore formation by *B. Welchii*. As has been established within recent years by the work of Bogendorfer (1922), Gorke (1922), van der Reis (1925), Syderhelm (1922) and others, with the absence of gastric acidity a flora tends to appear in the small intestine which is in many respects qualitatively and quantitatively like that of the large bowel. The content of the normal large intestine has generally a reaction about

[2] Our thanks are due to Dr. M. Lake for these specimens obtained from patients in the gastro-enterological clinic of Cornell Medical College.

neutral or slightly acid. This is true, also, for cases of gastric achlorhydria if we may judge from our determinations of the stool reactions. In cases of gastric anacidity it would follow, then, that the site of active spore formation of *B. Welchii* is probably the small rather than the large intestine because in the former *B. Welchii* first meets reaction conditions which our *in vitro* experiments indicate are optimal. In this respect we are in accord with the conclusions of Nye, but we do not agree with his view that actually there would be little difference in the total intestinal counts of vegetative *B. Welchii* in normal individuals and those with gastric achylia or in other words that there is no more active growth of *B. Welchii* in the latter than in the former. He arrived at this conclusion because enumerations of vegetative *Welchii*-like rods, as seen in gram-stained fecal films, gave approximately the same counts for normals and cases with achylia (including 6 pernicious anemia patients). His spore counts indicated, however, almost 200,000 times as many per gram of dried fecal material from pernicious anemia cases as from normals. Is it not fair to assume that this excess indicates more active growth of *B. Welchii* at some intestinal levels in the former? We believe that such is the case, for it is obvious that each spore must have been preceded by a vegetative form. The only other explanation of the count discrepancies would seem to be that in achylia and pernicious anemia cases the intestine is seeded with more spores through food ingested than in normals but that of course is an unreasonable assumption. The truth of the matter seems to us to be that in conditions of achylia gastrica the small intestine is seeded with the flora of the large bowel and that the reaction in the latter is favorable not only for the spore formation but also for the growth of *B. Welchii*. Davidson (1928) has recently confirmed Nye's findings as regards *B. Welchii* spore counts in the stools of pernicious anemia cases as compared with normal subjects. As stated above, however, we are in entire agreement with Davidson's conclusion that this figure is indicative of that amount of actual increase of the vegetative organisms at some higher level of the intestinal tract or, in other words, that a much more active multiplication of

B. Welchii occurs in the intestine of pernicious anemia cases than in that of normal individuals.

SUMMARY

1. Through the use of a sugar-free, well buffered fluid medium, in which a minimum reaction change occurs as a result of the growth of *B. Welchii*, it has been shown that the zone favorable for spore formation by this organism ranges from pH 6.8 to the alkaline limit for its growth with an optimal reaction between 7.4 and 8.0.

2. Spore formation is entirely checked during the first week in reactions more acid than pH 6.6.

3. We have not found that mannitol and raffinose in the medium favors spore formation as claimed by Noguchi and also Fitzgerald.

4. The high *B. Welchii* spore counts for fecal specimens from cases with gastric anacidity (including pernicious anemia) may be explained by the fact that the reaction of the small intestine in such cases is favorable for both spore formation and active growth of this organism.

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BACTERIAL SPOILAGE OF A THOUSAND ISLAND DRESSING

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INTRODUCTION

The commercial preparation of mayonnaise and similar dressings is one of the newer industries which has arisen from a greater knowledge of nutrition. With the discovery of vitamines in green vegetables and the subsequent increased consumption of lettuce, celery, cabbage, asparagus and other vegetables, came the need of a dressing to make them more palatable. The French originated this sauce and it had been prepared in the home with more or less success. Preservation of the product has not been an important factor in the home-prepared dressings since they are ordinarily used shortly after preparation. The lack of knowledge of the value of egg yolk in stabilizing the emulsion and of vinegar in preservation, the use of proper amounts of seasoning and the proper ratio of the various ingredients has proved a serious handicap to the commercial development of this industry, but in spite of this, production has increased by leaps and bounds.

Until recently the problem of stabilizing the emulsion attracted more attention than the problem of preservation. Since dressings of this nature are altered by heating, they cannot be preserved by sterilization and other methods must be practiced. Further complications ensue when mayonnaise is mixed with materials such as chili sauce, pickles, olives, pimentos or ground meat in the preparation of Thousand Island dressings, sandwich spreads and similar materials. The original preservative material may have been of sufficient concentration to inhibit the growth of

bacteria, but for example, when diluted with chili sauce, growth of the bacteria present in the mayonnaise may follow. Such a condition was called to our attention recently and it was the purpose of this work to discover the cause, note the effect and eliminate the spoilage.

HISTORICAL

The separation of oil and occasional subsequent fermentation has been noted since the industry began. Iszard (1927) examined about fifty samples and noted three stages in the spoilage. Gas bubbles appeared first, followed by a rancid odor of butyric acid and finally by a separation. After separation organisms sometimes develop. The majority of these samples were fermented by the aerobic spore formers *B. petasites* Gottheil or *B. teres* Neide. The organisms could be isolated from the foundation paste but since this could not be sterilized, she studied the comparative value of lactic acid and vinegar as preservatives.

Bachmann (1927) studied a number of commercially prepared dressings finding spore bearing rods similar to *B. subtilis* Cohn and *B. mesentericus* Trevisan, as well as molds, micrococci and diplococci but no toxin producing anaerobes. In no case were all of the samples from any one manufacturer sterile. A close relationship was found between the number of organisms present and the acidity and added starch paste. The eggs were considered to be the source of the organisms.

Rettger (1914), Hadley and Caldwell (1916) and others have found that the majority of fresh eggs are sterile, but aerobic spore formers have been noted.

Gray (1927) found in pimentos a spore former which was heat-resistant to a considerable degree. He suggested sterilization of the separate ingredients, especially those added to mayonnaise to make sauces.

Bitting (1928) in studying the stability of the emulsion, noted that as the proportions of the ingredients approached the stable point, the loss due to growth of organisms was lessened.

THE FERMENTED DRESSING

The spoiled dressing which was studied was composed of a mayonnaise base made from corn oil, vinegar, egg yolk, sugar, paprika, mustard and pepper to which was added pickles, olives and chili sauce. The product was ordinarily a smooth, heavy-bodied emulsion of a light orange color. The acidity as determined by titrating with N/10 NaOH using phenolphthalein as an indicator was 0.71 per cent as acetic acid. The hydrogen ion concentration was between pH 4.2 and 4.4.

The spoiled material was darker in color and showed separation of oil in varying degree. The oil could not be incorporated satisfactorily by beating. The titratable acidity had increased, one sample titrating 0.91 per cent acid. The hydrogen ion concentration had also increased to pH 4.0 to 4.2. The volatile acidity had increased from 0.55 per cent in the normal to 0.60 to 0.64 in the spoiled dressing.

Microscopic examination showed the presence of a large number of spores in all the samples of spoiled material examined. Apparently only one species was present. Transfers made into yeast extract agar slants showed abundant growth in twenty-four hours but no spores until the second day. Several of the samples were plated, isolations made, and the organism studied in detail. Only one type of colony developed upon the plates.

CHARACTER OF THE CAUSATIVE ORGANISMS

The morphological and cultural characters are as follows:

Morphology. The organisms are from 0.5 to 0.8 microns by 1.5 to 3.0 microns long on glucose agar. Chains up to 10 microns in length have been noted in very young cultures, but ordinarily organisms occur singly. On potato slants and in gelatin stab, the organisms are somewhat larger. They are actively motile.

Staining properties. They are Gram-positive.

Spore formation. Spore formation begins after twenty-four hours on glucose agar. Spores are usually central, but occasionally eccentric. Spores measure from 0.5 to 0.6 microns by 0.7 to 1.0 microns. The protoplasm at the ends when attached may

shrink giving the appearance of a bulging cell. The protoplasm ordinarily remains attached to the spore.

Growth on agar slant. Growth on agar slants is abundant, heavy white and wrinkled. During the first twenty-four hours the growth appears moist and glistening, but later it becomes dry and dull. The growth is membranous and lifts easily from the agar. A decided odor is noted.

Growth in agar stab. The growth is heavy and wrinkled on and near the surface, but very faint further down along the line of inoculation.

Agar colonies. Colonies after two days are spreading, white, ameboid with edges entire. The surface is finely granular.

Growth in broth. A pellicle forms on broth, the liquid becoming slightly cloudy.

Growth in gelatin stab. Growth is rapid, and liquefaction complete in two days at 18°C.

Growth in litmus glucose agar stab. On litmus glucose agar stab a heavy surface growth appears with a faint growth along the line of inoculation. The medium becomes alkaline with a slight reduction at the bottom. Later the medium becomes alkaline from the top toward the bottom.

Growth on potato. Growth on potato is profuse, spreading and slimy with a heavily wrinkled surface. Growth is grayish, and the potato is discolored to a brown or pink.

Indole production. Negative.

Reduction of nitrates. Nitrates are reduced on peptone or synthetic agar slants.

Growth in litmus milk. The milk is peptonized completely. The color becomes purplish.

Growth in glucose broth in fermentation tubes. The growth is luxuriant with a pellicle in the open arm. No gas is produced.

Growth in carbodydrates and related compounds. Acid is produced in glucose, fructose, lactose, maltose, sucrose, raffinose, rhamnose, inulin, dextrin, starch, glycerol, mannitol, sorbitol and salicin, but not in xylose. The hydrogen ion concentration attained is from pH 5.6 to 6.0.

This description is similar to that of *Bacillus vulgatus* Trevisan as given by Bergey (1930) or by Ford (1927).

THE SOURCE OF CONTAMINATING ORGANISMS

When this problem was called to our attention it was believed that the chili sauce contained the contaminating organism. This was the natural conclusion, since the spoilage did not occur when the chili sauce was omitted in the preparation of dressings. With this in mind, microscopic preparations were made and examined, the chili sauce was plated on various types of agar, and inoculated into various broths, but no sign of living organisms could be noted. Samples were then obtained of the other ingredients of the dressing.

Since Bachmann (1927) believed the eggs to be the source of contamination and Rettger (1914) showed the presence of aerobic spore formers in eggs, the frozen egg yolk was next examined. The product appeared to be normal in every respect. Microscopic examination showed the presence of non-spore forming, Gram-negative rods, but no spores or Gram-positive rods. Knowing that if the spore formers were present they would be there in small numbers an attempt was made to kill off the non-spore forming bacteria and then by plating to recover the spore formers. With this in view 5 grams of the egg yolk were diluted with 100 cc. of water heated for thirty minutes at 150°F. and then plated. The colonies found developing upon these plates were few in number and of one type. Microscopic examination showed the organisms to be Gram-negative, short rods. The organisms produced abundant acid and gas in carbohydrate media, reduced nitrates and formed indole. They failed to form spores. If any spores were present in the egg yolk, the characteristic colonies should have formed on the plates, but none of the colonies showed any resemblance to the colonies ordinarily formed by true bacilli.

It did not seem to be possible that the spices could be the source of the contamination, since Bachmann (1916) showed that spices have some preservative value especially in concentrated form. Cutter (1922) found *Cl. botulinum* Holland growing in an acid tomato onion sauce. Bachmann (1923) found the same organism in a highly spiced mince meat and Albus and Ayers (1928) found that pimentos may have been the cause of gassy fermentation in

processed cheese. With these facts in mind a thorough study of all the ingredients was made.

Approximately 0.1 gram of pepper, mustard, paprika, salt and sugar and 1 cc. of vinegar were plated directly using a peptone glucose agar. The three latter materials failed to produce colonies in two days at 25°C., but colonies developed on the plates from pepper, paprika and mustard. Only two colonies developed on the mustard plate, one a colon-type colony, the other similar in every respect to those from the dressing. The number of these colonies was not great, but since they spread so quickly, the plates were practically covered. Organisms were isolated from these plates. Comparative studies showed that the bacteria in the pepper and the paprika were identical with those previously isolated from the dressing. The organisms from the mustard showed but slight differences which suggests that they were a closely related species.

STUDY OF OTHER SEPARATED DRESSINGS

Other samples of separated mayonnaise, Thousand Island dressing, sandwich-spread and Russian dressing were studied in a similar manner. With the exception of a sandwich-spread, none of these materials showed an appreciable number of organisms and even after standing several months at room temperature no further change was noted. The separation was therefore believed to be due to an unstable emulsion.

The sandwich-spread showed a number of spore formers present. Morphological and cultural studies of this organism showed it to be similar to, but not identical with, those previously described. Not all the ingredients were available for study in this case and therefore it was impossible to make a more complete study. Of those materials available the corn starch was the only one which contained spores of bacteria.

METHODS OF PREVENTING LOSSES

Since it is impossible to sterilize the final product, some other method of preventing losses becomes necessary. Two methods of

attacking this problem are possible. In one, the acidity of the product may be increased so that organisms can not grow. This may be accomplished by the use of lactic acid as shown by Iszard (1927). It may also be accomplished by the addition of more vinegar, but difficulties may arise in that the emulsion may be unstable if too much vinegar is added as shown in the work of Bitting (1928). In certain cases, an acidity high enough to inhibit the growth of such organisms as these spore formers is undesirable.

A second method of attack is to kill the organisms before the contaminating material is added to the product. In this case this would be the pepper and paprika, and possibly the mustard.

A test was carried out in which the pepper, the paprika and the mustard were placed in vinegar, allowed to stand five minutes and then plated. The vinegar was then brought to a boil, the boiling continued for five minutes, samples being taken for plating at one minute intervals. Samples boiled for one minute showed a decided reduction in number of organisms and after three minutes no growth appeared in any of the samples. This method of sterilization could, of course, be applied to certain of the ingredients of the dressing only.

CONCLUSION

An outbreak of spoilage of Thousand Island dressing was found to be caused by an aerobic spore former, *Bacillus vulgatus* Trevisan.

The contaminating organisms were found to come from the pepper and the paprika used in the dressing.

Methods of sterilization may be of two types: (*a*) preparing a product of such acidity that it will not allow the growth of organisms, or (*b*) sterilization of the ingredient which contains the contaminating organisms.

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A STUDY ON THE DISSOCIATION OF THE DIPHTHERIA BACILLUS

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Comparatively little work has been done on the dissociation of the diphtheria bacillus into smooth and rough forms, despite the fact that virulent and non-virulent strains of the diphtheria bacillus have been described as long ago as Roux and Yersin's work in 1890. In 1897 Corbett and Phillips actually showed that a pure line culture of the diphtheria bacillus yielded two types of colonies, one virulent and the other non-virulent. In 1898 Slawyk and Maincatide may well have been describing smooth and rough colonies when they stated that among 30 strains of the diphtheria bacillus they found one kind of colony which was convex and glistening and another kind which was flat and dull. It was not, however, until nearly thirty years later that it was shown that virulence was associated with the smooth type of colony, and non-virulence with the rough type. Cowan in 1927 showed that a stock culture of the Park 8 strain split into a smooth, virulent and a rough, non-virulent form.

In the present study an attempt has been made to answer the following questions:

1. Is virulence always associated with the smooth form of the diphtheria bacillus and non-virulence with the rough form?
2. Is toxin formation related to the morphological difference in the colonies?
3. Is it possible to transform at will the smooth form into the rough form, and *vice versa*? If so, under what conditions does this transformation take place?

4. Which forms are found in the acute stage, the convalescent stage and the carrier stage of diphtheria?

5. Do the rough forms possess any immunizing value?

In addition, it was kept in mind that the objection might be raised that rough, non-virulent bacilli found in throat cultures, although they might resemble diphtheria bacilli under the microscope and even in fermentation reactions, might not be true diphtheria bacilli. This objection is difficult to meet conclusively, but if it were possible to show that rough forms could be derived in the test tube from a pure, smooth culture, and that these rough forms were similar in every respect to those found in throat cultures, it would be very strong evidence that the rough forms occurring in the throat were genuine diphtheria bacilli.

MATERIAL

With the above problems in mind, the study was commenced by securing throat cultures from diphtheria cases at different stages of the illness.

The surface growth on the Loeffler slant was washed off with about 5 cc. of hormone broth and gently emulsified. A loopful of the emulsion was then streaked on each of two blood agar plates. The blood agar plate was used because it was easy to pick out the colonies of diphtheria bacilli from those of streptococci and pneumococci. The plates were carefully examined the next morning, after about twenty hours' incubation, under the stereoscopic binocular microscope. Diphtheria-like colonies were picked out and replated on blood agar plates. After the second plating, colonies—whether rough or smooth—which showed characteristic diphtheria bacilli were transferred to Loeffler's slants, and the fermentation reactions and virulence tested.

In the acute stage, 47 throat cultures were studied, and all showed smooth colonies only.

In the convalescent stage, among 79 cultures 16 were found with both rough and smooth colonies.

In the carrier stage, the 3 cases that were investigated showed both types of colonies.

Thirty-one cases of clinical diphtheria were cultured system-

atically up to the time of their release. In the acute stage, no rough forms were found, but in 5 cases the cultures for release showed a few rough colonies.

Lastly, a culture of the Park-William 8 strain that was being used for toxin production was studied carefully. Colonies were found which appeared to be intermediate in type. They had a granular surface, but were not so rough as the rough forms found in throat cultures.

Examination of material

The various strains that had been obtained from these sources were examined for their morphological and cultural characteristics, their fermentation reactions, their virulence, their toxin production, and their immunizing properties.

Morphology and cultural characteristics. The smooth forms are generally thinner, longer and more striated than the rough forms, which are apt to be somewhat short, plump, and to stain uniformly. The smooth colonies are glistening and whitish in colour, while the rough colonies are smaller, denser, more granular and greyish in colour. The smooth type forms a thick pellicle on broth with little growth at the bottom of the tube, while the rough form, when freshly isolated, forms no pellicle at all and tends to grow at the bottom of the tube. On passage in broth a thin pellicle is ultimately formed. The surface growth on a Loeffler slant of the smooth organism can be emulsified into a uniformly turbid suspension, but the rough form, similarly treated, gives a granular suspension.

Fermentation reactions. There has been considerable discussion as to the reliability of fermentation in differentiating the diphtheria bacillus from the diphtheroids. However, many reliable workers, including A. Knapp (1904), Hans Zinsser (1907), J. G. Fitzgerald (1924), C. C. Okell and Baxter (1924), working with a great number of strains of diphtheria bacilli as well as with diphtheroids, have come to the conclusion that the fermentation test does differentiate the Klebs-Loeffler bacillus from the other organisms. Two-hundred and sixteen cultures of diphtheria bacilli were studied, of which 164 were smooth forms and 52 were

rough. These, as well as cultures of *B. hoffmanni* and *B. xerosis*, were tested in serum water media containing glucose, dextrin and sucrose. The tubes were read seven days after inoculation. All the diphtheria bacilli formed acid in glucose and dextrin serum

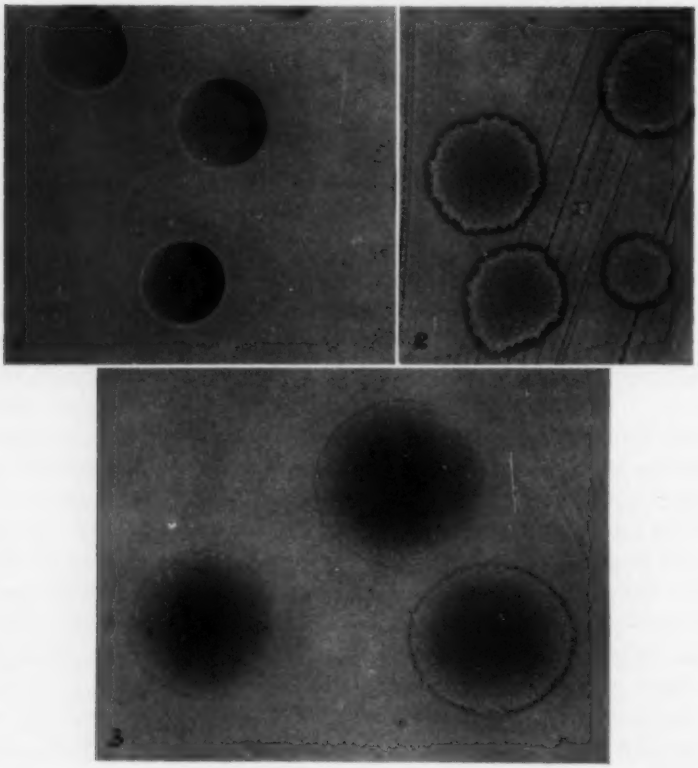

FIG. 1. SMOOTH TYPE COLONIES
FIG. 2. ROUGH TYPE COLONIES FROM THROAT CULTURE
FIG. 3. INTERMEDIATE TYPE COLONIES FROM PARK 8 STRAIN

water, but not in sucrose. The rough forms gave the same fermentation reactions as the smooth forms.

Virulence. In testing for virulence, the various strains were grown for twenty-four hours on Loeffler's medium. The growth

on the slant was washed off in 10 cc. of saline and 0.1 cc. of this emulsion was injected intradermally into each of 2 guinea pigs, one normal and the other protected with 500 units of antitoxin. In general, 5 strains were tested on each pair of guinea pigs. Two-hundred and sixteen strains were tested in this way with the following result: 164 strains were smooth and 146 of these were virulent and 18 avirulent; the 18 avirulent smooth forms were all obtained from convalescents or carriers; 52 strains were rough and all were avirulent.

Toxin production. In testing for the production of toxin, the procedure was as follows: Cultures were transplanted daily for three days in broth, and then a piece of pellicle was inoculated into 40 cc. of special broth contained in a 300 cc. flask. The special broth was prepared according to Harrison's formula, from the United States Hygienic Laboratory. The initial pH of the broth was 7.3. The inoculated broth was incubated for five days at 36°C., the reaction of the broth being checked daily. At the end of the incubation, the culture was centrifuged, the supernatant fluid filtered through a Berkefeld "N" filter, and the filtrate tested for toxicity by guinea pig injection. Twelve rough strains and 5 avirulent, smooth strains were found to be completely atoxic. The cultures remained acid during the incubation period, and 5 cc. of the filtrates had no effect whatever on guinea pigs, when injected subcutaneously. Five smooth, virulent strains were tested. Two out of the 5 produced a toxin with a M.L.D. of 0.004 cc. The remaining 3 strains, although virulent, produced a toxin with a M.L.D. of 1.0 cc. It is interesting that these 3 strains were isolated from the throats of 3 different carriers. The toxicity of the cultures of the 2 types of colonies isolated from the Park 8 strain was tested intracutaneously on guinea pigs. The filtrates were first diluted 1:500 and then titrated (table 1).

Table 1 shows that the smooth colony type of Park 8 strain produces a toxin at least 5 times stronger than the toxin produced by the intermediate colony type.

Immunizing properties. Guinea pigs were injected both subcutaneously and intraperitoneally with filtrates of cultures of the

rough colony organisms. Three weeks after the last injection of rough filtrate, the guinea pigs were tested with 1 M.L.D. of toxin. All the animals died within six days. The rough filtrates, then, produce no antitoxic immunity whatever.

The experiment was repeated, using the filtrates of smooth avirulent organisms. Again no immunity resulted.

As was to be expected, the injection of living, rough bacilli did not confer any immunity on guinea pigs, the subsequent injection of living, smooth, virulent organisms being fatal to these animals.

TABLE 1

AMOUNT OF FILTRATE	1:500 FILTRATE FROM PARK 8 SMOOTH COLONY	1:500 FILTRATE FROM PARK 8 INTERMEDIATE COLONY
cc.		
0.125	++++	++++
0.08	++++	++++
0.04	++++	+++
0.01	++++	++
0.005	++++	0
0.0025	+++	0
0.0015	++	0
0.001	0	0

IN VITRO EXPERIMENTS ON THE DISSOCIATION OF THE DIPHTHERIA BACILLUS

1. Attempt to dissociate the "S," or smooth form, into the "R," or rough form, by means of incubation at a temperature of 45°

Hewlett and Knight (1897) reported that virulent diphtheria bacilli were changed into avirulent vacilli by seventeen hours' exposure at 45°C. Accordingly, 5 smooth, virulent toxic strains were incubated at 45° for seventeen hours for 20 passages, plating out on blood agar plates and incubating the plates at 37°C. between each 45° cultivation. Examination of the plates usually showed only a few scattered colonies, but these were as smooth and glistening after the twentieth passage as were the original colonies. A Loeffler slant at 36°C. after the tenth passage, however, was completely avirulent for guinea pigs. Examination of all the strains at the end of the experiment showed that they were

all atoxic as well as avirulent. Hewlett and Knight's work was thus confirmed, but no dissociation into the "R" form had taken place under these conditions.

2. Attempt to dissociate the "S" form into the "R" form by means of cultivation in antitoxic serum

Observations on the transformation of toxic and virulent strains of diphtheria bacilli into atoxic and avirulent strains have been made by Bernhardt (1916), Levinthal (1926), Becker (1927), and recently by Jungeblut (1928). Jungeblut, after cultivating 2 strains of diphtheria bacilli in broth containing normal horse serum on the one hand and antitoxic serum on the other, found that the cultures in normal horse serum broth remained unchanged, but that the cultures in the antitoxic serum broth lost their capacity to produce toxin after 6 passages in this medium, while retaining their virulence of guinea pigs.

Five strains of smooth, virulent, toxic bacilli were cultivated in 3 different media.

a. 1.0 cc. normal horse serum + 4.0 cc. of broth.

b. 1.0 cc. of concentrated antitoxic serum (10,000 units per cubic centimeter) + 4.0 cc. of broth.

c. 1.0 cc. of unconcentrated antitoxic serum (50 units per cubic centimeter) + 4.0 cc. of broth.

After 10 passages, none of the strains in any of the 3 mediums showed any colony difference; so they were then cultivated in undiluted normal horse serum and undiluted antitoxic serum for another 10 passages. Still the appearance of the colonies was unchanged, being perfectly smooth and glistening, while the morphology of the bacteria also remained normal. Cultures from both the normal serum and the antitoxic serum were tested at the end of the experiment for virulence and toxin production. Both sets of cultures were virulent, and the M.L.D. of the toxin produced was about 0.02 cc.

No dissociation into the "R" form occurs, then, if "S" strains are cultivated in antitoxic serum.

3. Attempt to dissociate the "S" form into the "R" form by means of cultivation in anti-bacterial serum

The method used for the production of anti-bacterial serum was similar to that used by Fitzgerald and Doyle (1923) in making agglutinating serum for *B. diphtheriae*, except that the cultures were washed 3 times instead of 14 times, as in Fitzgerald and Doyle's technique. A suspension of the washed bacilli of 10 strains of smooth virulent organisms was made up and injected into rabbits in the following way:

a. 1.0 cc. of the suspension was heated to 60°C. and injected subcutaneously.

b. One week later, 2.0 cc. of the heated suspension was injected subcutaneously.

c. One week later, 2.0 cc. of the unheated suspension was injected intravenously.

d. One week later, 5.00 cc. of the unheated suspension was injected intravenously.

e. One week later, 10.0 cc. of the unheated suspension was injected intravenously.

Rabbits were bled seven days after the last injection. The sera so obtained usually agglutinated in a concentration of 1:3000. They also contained a small amount of antitoxin—about 2.0 units per cubic centimeter.

The serum of one such rabbit was distributed into small tubes, each tube containing 0.5 cc. of the undiluted serum. A similar set of tubes, each containing 0.5 cc. of normal rabbit serum was prepared at the same time. Five strains of smooth, virulent toxin-producing diphtheria bacilli, together with a strain of *B. xerosis*, were cultivated in the anti-bacterial serum and in the normal serum. Sub-cultures were made every other day and plates streaked at the same time. On the tenth passage a few rough colonies were observed in 2 out of the 5 strains. These were replated and found to produce rough colonies only. The other 3 strains were then transferred to the serum of another immunized rabbit and cultivated as before. After the sixth passage a few rough colonies were seen in one strain, and the number of rough

colonies increased with the number of passages until, at the twentieth passage, there were an equal number of rough and smooth colonies. The remaining 2 strains and the culture of *B. xerosis* underwent no change.

The rough colonies were tested and found to conform in every way to those found in the throats of convalescents. The morphology of the bacilli and the appearance of the colonies were indistinguishable from those of the rough forms described earlier in this paper, the fermentation reactions were typical, the strains were totally avirulent and atoxic and they produced no immunity. No explanation was found to account for the fact that 2 of the strains cultivated under these conditions remaining unchanged during 30 passages.

4. Attempt to change the "R" form back into the "S" form by animal inoculation

One rough strain, transformed from the smooth form by means of cultivation in anti-bacterial serum, and the Park 8 intermediate form were used. The organisms were grown separately in 100 cc. of broth, the cultures were then centrifuged and the sedimented bacilli resuspended in 2 cc. of saline. Each suspension was injected into a guinea pig subcutaneously. With the rough organisms an abscess formed at the site of injection, but cultures of the pus showed pure cultures of the "R" form. The guinea pig injected with the Park 8 strain died and cultures showed intermediate forms only.

5. Attempt to change the "R" form back into the "S" form by cultivation in "R" antibacterial serum

For this experiment serum was prepared in the same way as with the "S" anti-bacterial serum, but rough bacilli were used instead of the smooth, virulent organisms, and the suspensions were not heated for any of the injections. Two rough strains were cutivated in this "R" anti-bacterial serum and showed no change after 15 passages.

DISCUSSION

In the light of the investigations and experiments that have been recorded above, the questions that were posed in the beginning of this paper can now be discussed.

Is virulence always associated with the smooth form of the diphtheria bacillus and non-virulence with the rough form? The answer to the first part of the question is in the negative, as many typically smooth forms were found that lacked virulence, although it is significant that these smooth, avirulent forms were only found in the convalescent stage and not in the acute stage of the disease. Whether they are, or are not intermediate forms between the smooth and the rough forms is not known at present. The answer to the second part of the question is in the affirmative, as was of course to be expected. All the rough forms tested were completely avirulent. This is in conformity with the most important characteristic of the "R" forms of the majority of pathogenic organisms.

Is toxin formation related to the morphological difference in the colonies? The answer to this question is closely parallel to what has been said about virulence. The rough forms are always atoxic, but some of the smooth forms produce no toxin. It is interesting that in the throats of carriers, smooth forms were found which, although virulent by the usual test, formed only a very feeble toxin. Attention has been called to the fact that the Park 8 strain produces a powerful toxin and yet is not very virulent, so that the reverse picture described here is additional evidence that there is another factor in virulence beside the ability to produce a strong toxin.

An intermediate form was found in studying a culture of the Park 8 strain. This was not a glistening, smooth colony, nor was it a typical rough. It produced a toxin which, although fairly strong, was yet very definitely weaker than that produced by the smooth Park 8 colony.

Is it possible to transform at will the "S" form into the "R" form and *vice versa*? If so, under what conditions does this transformation take place? The loss of virulence or the capacity

to produce toxin has been reported many times in the literature, and in some cases this has been brought about by subjecting the virulent, toxic bacilli to an abnormal cultural environment. The experiments described in this paper, however, show that this loss in biological activity is not accompanied by any change in morphology or colony formation. Apparently the only condition which results in the transformation into the "R" form is the presence of specific anti-bacterial serum in the culture medium and even strong, undiluted antitoxic serum does not bring about this change. "R" forms occur in the throats of convalescent cases, and it would thus seem probable that the *in vivo* transformation is due to the anti-bacterial antibodies in the patient's tissues and not to the antitoxin antibodies. Carriers have been treated by the injection of diphtheria bacilli vaccines in order to rid the throat of the organisms, so far with very little success, but this treatment should have raised the anti-bacterial content in tissues and it is at least possible that the bacilli which persisted after the treatment were rough forms and therefore innocuous.

Experimentally it has been proved that the rough, avirulent, atoxic diphtheria bacillus is a variant of the smooth, virulent, toxic organism, but it has not been possible either *in vitro* or *in vivo* to change the "R" form back into the "S." Although this reversion cannot be demonstrated, there is always the possibility that it may take place in the human body under conditions of which we are at present ignorant.

Which forms are found in the various stages of the disease? In the acute stages only the "S" forms are found. The "R" forms begin to make their appearance during convalescence and this is presumably the result of the elaboration of anti-bacterial antibodies in the patient. Avirulent "S" forms also occur later in the disease and it is possible that this change may be brought about by the antitoxic antibodies.

Do the rough forms possess any immunizing value? As was to be expected, the "R" forms have no immunizing value whatever.

The "R" forms, which were derived from pure cultures of the "S" forms *in vitro* resembled in every particular the "R" forms isolated from convalescent diphtheria cases and carriers, and this

constitutes very strong evidence that the latter forms were genuine diphtheria bacilli and not diphtheroids.

SUMMARY

1. Forty-seven cultures of diphtheria bacilli isolated from patients suffering from diphtheria showed smooth, virulent forms only. Of 79 throat cultures from diphtheria patients during the convalescent stage, sixteen cultures showed the presence of both smooth and rough forms of diphtheria bacilli.

2. Thirty-one cases of clinical diphtheria were cultured at different stages, from the onset of illness up to release. Not a single rough form was found during the acute stage, but 5 cases showed the presence of rough types of diphtheria bacilli from cultures for release.

3. In 3 cases of carriers which were studied, the rough forms of diphtheria bacilli formed the predominating type.

4. One culture of Park 8 diphtheria bacilli was dissociated into smooth and intermediate types by repeated plating on blood agar plates, and while both forms produced toxin, the smooth type produced a much more potent toxin than did the intermediate type.

5. Sugar fermentations give reliable data for the recognition of diphtheria bacilli, but do not differentiate the smooth from the rough type, nor the virulent bacilli from the avirulent.

6. The virulence of 216 strains of diphtheria bacilli was tested. Fifty-two were rough forms and were all found to be totally avirulent and atoxic. One hundred and sixty-four were smooth in cultural characteristics but while 146 were virulent, 18 of them were avirulent. All the avirulent forms were isolated from patients during the convalescent stage or from carriers. These 18 cultures demonstrate that while loss of toxicity is usually associated with the cultural characteristics spoken of as "rough," a diphtheria culture may retain its smooth form and still have lost its toxic properties.

7. Toxicity tests were made on 12 strains of rough types of diphtheria bacilli and 6 strains of rough types transformed from smooth types. They were totally atoxic. Avirulent, smooth

forms were also found to be atoxic, while 3 strains of virulent, smooth forms isolated from carriers were toxic, although the toxin produced by them was weak.

8. Eighteen guinea pigs immunized with filtrates of the rough cultures of diphtheria showed no protection against the filtrates of toxic, smooth types of diphtheria bacilli.

9. Eleven guinea pigs immunized with cultures of the rough type of diphtheria bacilli showed no protection against the smooth types of virulent diphtheria bacilli.

10. By means of repeated exposure of virulent cultures to a temperature of 45°C. for seventeen hours the virulence can be reduced, but this treatment has no effect upon the appearance of the colonies.

11. Smooth, virulent diphtheria bacilli inoculated into diphtheria antitoxin-broth mixtures and then into pure antitoxin showed no change after 20 passages, while 3 out of 5 strains of diphtheria bacilli tested showed a change of smooth type to rough type after subculturing in anti-bacterial serum.

12. Various attempts were made to transform the rough type of diphtheria bacilli into the smooth type again by means of growing the rough type in the toxin-broth mixture, animal inoculation and subculturing in antibacterial serum of rough diphtheria bacilli. By none of the above mentioned methods was it found possible to convert the rough type back to the smooth type.

CONCLUSIONS

1. Smooth, virulent and toxic diphtheria bacilli are transformed to non-virulent, "R" forms in the throats of patients during convalescence.

2. The transformation may pass through an intermediate stage in which diminished toxin formation is not associated with morphological change.

3. *In vitro* experiments indicate that the dissociation "S" → "R" is governed by contact with anti-bacterial rather than with antitoxic serum.

4. The vaccination of carriers of "S" diphtheria bacilli with "S" vaccine is thus rendered logical.

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AN IMPROVED TECHNIC FOR THE VOGES-PROSKAUER TEST

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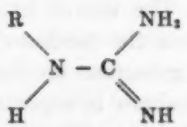
Voges and Proskauer (1898) described the production of an eosin-like coloration in glucose peptone cultures of certain organisms to which had been added a 10 per cent solution of potassium hydroxide. The reaction is generally referred to as the V-P test and serves as a valuable aid to the identification and differentiation of microörganisms.

The reaction depends upon the production of acetoin (acetylmethyl carbinol), a non-volatile reducing substance. According to Harden (1905) acetylmethyl carbinol is oxidized upon the addition of KOH and in the presence of peptone there is imparted an eosin-like coloration to the medium. Later Harden and Norris (1911) reported that diacetyl in the presence of strong KOH solution reacts with proteins to give a pink coloration with a green fluorescence. The coloration appears to depend on the group



although the significance of the radical R is uncertain. The Voges-Proskauer reaction may be indicated as follows:

$$C_6H_{12}O_6 \rightarrow CH_3 \cdot CO \cdot CHOH \cdot CH_3 + KOH \rightarrow$$

glucose — acetylmethyl carbinol

$$CH_3 \cdot CO - CO \cdot CH_3 + \text{peptone} \rightarrow \text{pink coloration}$$

diacetyl

Harden and Norris (1912) explained the production of acetylmethyl carbinol and 2,3-butylene-glycol by assuming a condensation of intermediately formed acetaldehyde. Subsequently Neuberg and Reinfürth (1923) showed that if acetaldehyde is added to a sugar-containing medium undergoing fermentation by yeast, acyloin condensation occurs, one molecule of added aldehyde uniting with one molecule of aldehyde produced by the yeast. The added aldehyde is thus converted practically completely into acetylmethyl carbinol. Neuberg sees in this aldehyde condensation, a carbon-coupling action of yeast under the influence of an enzyme which he terms carboligase.

The eosin coloration of the positive V-P test is apparent only after a period of an hour or longer, and in the case of a number of microörganisms is faint and indistinct, making reading of the test uncertain and unreliable, especially in colored media. Among the attempts which have been made to improve the method of conducting the test, may be mentioned that of West (1909) who conducted air through the medium to hasten the reaction. Chen and Rettger (1920) suggested shaking and incubating at 30°C. for one to three hours and again shaking. Attempts also have been made to add oxidizing agents to increase the rate of oxidation of the acetylmethyl carbinol to diacetyl. Levine, Weldin, and Johnson (1917) after a study of various oxidizing agents, recommended the addition of two or three drops of hydrogen peroxide to 6 cc. of the alkaline medium heated in a boiling water bath for two minutes. The use of hydrogen peroxide was not entirely satisfactory when the medium was colored as was the case when a glucose or galactose medium was used. The difficulty with a glucose medium is especially important since the V-P test applies particularly to a glucose medium.

With sucrose, mannitol, raffinose, and salicin, the hydrogen peroxide method was found to give results as good as were obtained by reading the standard test after twenty-four hours. Bedford (1929) used sodium peroxide, adding 10 mgm. sodium peroxide to 2.5 cc. of the culture medium. One cubic centimeter of 40 per cent NaOH was then added and the culture tube placed in boiling water for one minute and then shaken.

An important difficulty experienced in the use of oxidizing agents is the transitory nature of the coloration of the positive cultures. Any method which results in the rapid disappearance of the coloration is inconvenient and likely to prove unsatisfactory, particularly since the eosin coloration is rather delicate.

The method to be described utilizes ferric chloride as a catalyst and avoids the disadvantages of the original V-P test or its modifications. The positive test is indicated by a deep copper coloration which appears at the surface after a few minutes and extends to the bottom of the tube. The color remains for several days and even after a week or longer it is clearly visible. The test may be positive for cultures of organisms belonging to the genus *Aerogenes* after three days' incubation at 30°C., but four-day cultures are recommended for standard procedure. Two drops of a 2 per cent solution of ferric chloride are added to 5 cc. of the culture. Five cubic centimeters of a 10 per cent solution of NaOH are now added and the tube shaken. The solution of ferric chloride must be added before addition of the sodium hydroxide solution; addition after the alkali results in a marked flocculation.

The reaction may be accelerated by heating the culture for one minute in boiling water. The advantages are not great and it is doubtful whether heating is to be recommended. Only the original culture, or the culture with ferric chloride, should be heated. Addition of the alkali before heating may result in browning if unfermented glucose is present.

In the positive tube, two distinct layers of coloration may be observed. The upper is a copper color; the lower layer is the eosin pink of the standard V-P test. The copper color soon extends to the bottom of the tube. Acetylmethyl carbinol may be detected in three-day cultures when the standard technic fails to show its presence. Since the color of a positive test is that of bright copper, reading of the test is definite and certain. The color remains for days and sometimes as long as two or three weeks. There is no danger of failure to observe the positive test.

The ferric chloride hastens the oxidation of the acetylmethyl

carbinol to diacetyl which reacts with peptone in an alkaline solution to produce the copper coloration. The ferric chloride appears to catalyze the oxidation of the acetylmethyl carbinol since the reaction does not take place in the absence of a hydrogen acceptor. Thus the reaction does not occur in the absence of atmospheric oxygen if no other suitable hydrogen acceptor has been provided. Apparently, this is the first time an improvement of the V-P test has been sought in the use of a chemical catalyst. Former attempts have used oxidizing agents which resulted in rapid oxidation and the transitory appearance of the eosin coloration.

Ferric chloride will not result in the oxidation of 2,3-butyleneglycol under the conditions of the test, and any organism which may have reduced the acetylmethyl carbinol to 2, 3-butyleneglycol will not give a false Voges-Proskauer reaction.

To show this, a medium of 2,3-butylene-glycol[1] (0.2 per cent), $(NH_4)_2$ So_4 (0.2 per cent), K_2HPO_4 (0.1 per cent) sterilized for fifteen minutes at 20 pounds pressure, was prepared in tubes and inoculated with strains of *Aerobacter aerogenes*, *A. faeni*, *A. pectinovorum*, *A. motorium*, *A. mitificans* and *A. indologenes*. Acetylmethyl carbinol was detected in cultures of all the organisms on the second day by the use of ferric chloride. Uninoculated tubes to which ferric chloride and KOH had been added did not give positive tests. Cultures from which atmospheric oxygen was excluded by boiling the constituents were negative.

This modification has been used during the past three years in comparison with the standard procedure by graduate students on over 600 cultures, some negative, others positive, and no discrepancies have been found. More than 40 species of bacteria have been represented in these tests.

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THE GROWTH OF BACTERIA IN A CONTINUOUS FLOW OF BROTH

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While we are in the habit of restricting the conception of a bacterial colony to aggregates of cells on solid media, growth in fluid media follows the same general course and for purposes of comparison may be considered as a colony.

The growth curve for a colony of bacteria in a fluid medium follows very closely that of a colony of multicellular organisms up to the time when the maximum number is approached. When the normal population is reached in the usual colony of multicellular organisms it continues approximately level with only a slow increase or decrease, while the bacterial colony soon passes into a period of rapid decline. The analogy between the two kinds of colonies is not perfect because of the difference in conditions. In a colony of multicellular animals there is a constant supply of food, and natural or artificial provision for the removal of the products of metabolism. In the bacterial colony the quantity of food is limited and the products of growth accumulate. If the bacterial colony should be maintained under conditions which would permit a continuous supply of nutritive material and the removal of the products of growth, would the population be limited to its normal number and would it maintain itself indefinitely?

It is well known that colonies of bacteria do maintain themselves indefinitely, under certain natural conditions. An infected tonsil or a pus sac on a dead tooth harbor colonies of bacteria which may persist for years, and in which it is probable that the bacterial population is fairly constant.

Experiments designed to answer these questions were made in a culture flask (fig. 1) holding about 75 cc., provided with an inlet tube into which broth from a supply flask flowed. The rate of flow was regulated to about one drop per minute. This rate was

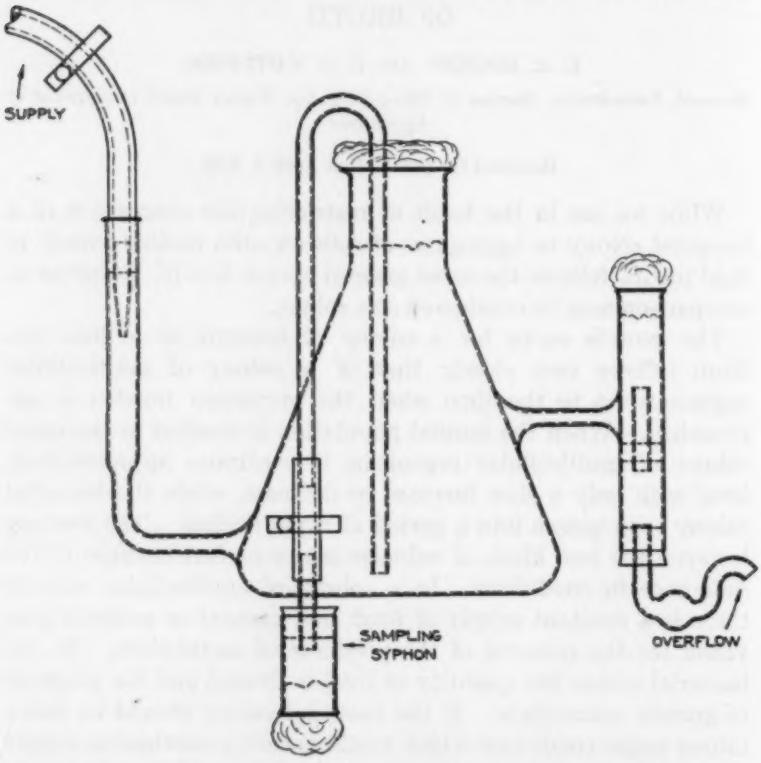

FIG. 1. APPARATUS USED IN STUDYING THE GROWTH OF BACTERIA IN A CONTINUOUS FLOW OF BROTH

sufficient to displace the medium in the flask once in 24 to 30 hours. Since the tube from the supply flask projected into the center of the inlet tube there was no chance for growth to work back into the supply flask. An overflow tube projected into a large vertical tube plugged with cotton at the top and trapped at

the bottom. Samples were taken from the culture flask by a siphon protected at the outlet by a tube plugged with cotton.

The broth contained only a trace of sugar to obviate the inhibiting effect of a high hydrogen-ion concentration.

In table 1 are shown the results obtained when a culture of *Str. lactis* was grown under these conditions at 30°C. In this experiment the population was maintained at a normal level for many days. On the sixteenth day, a failure of the regulating

TABLE 1

Str. lactis in continuous flow of low sugar broth

AGE	FLOW OF BROTH PER CENT OF CAPACITY OF FLASK	BACTERIA PER CUBIC CENTIMETER
days		
1	100.0	1,160,000,000
2	62.2	1,380,000,000
3	71.1	1,250,000,000
4	77.7	
5	57.7	905,000,000
6	80.0	
7	80.0	1,060,000,000
8	71.1	990,000,000
9	60.0	1,165,000,000
10	84.4	1,085,000,000
11	88.8	1,270,000,000
12	26.6	625,000,000
13	82.2	
14	82.2	
15	82.2	760,000,000
16	1,111.1	1,265,000,000

device caused all of the broth in the supply flask to flow through the culture flask in one night so that the medium was changed 11 times in a few hours. The count made the following day showed a normal population but, since some hours had elapsed, it does not necessarily follow that this number had been maintained throughout the rapid flow.

Similar results, shown in table 2, were obtained with *Esch. coli* which maintained a constant population in flowing broth for 30 days.

All of our previous experiments on the effect of *Str. lactis* and

Esch. coli on each other would lead us to expect that these two organisms would not grow equally well in the same flask. Table 3 gives the results obtained when they were inoculated together in a flask through which a low sugar broth was flowed slowly for

TABLE 2

Esch. coli in continuous flow of low sugar broth

AGE	FLOW OF BROTH PER CENT OF CAPACITY OF FLASK	BACTERIA PER CUBIC CENTIMETER
days		
1	78.5	870,000,000
2	89.2	1,255,000,000
3	71.4	1,730,000,000
4	64.2	
5	89.2	3,500,000,000
6	96.4	4,950,000,000
7	92.8	2,300,000,000
8	78.5	3,850,000,000
9	92.8	2,095,000,000
10	89.2	
11	71.4	
12	92.8	3,900,000,000
13	96.4	3,300,000,000
14	64.2	3,200,000,000
15	42.8	3,200,000,000
16	85.7	8,800,000,000
17	85.7	3,600,000,000
18	64.2	
19	107.1	2,730,000,000
20	71.4	2,955,000,000
21	71.4	1,645,000,000
22	78.5	3,000,000,000
24	132.1	1,480,000,000
25	28.5	
26	89.2	1,715,000,000
27	60.7	2,190,000,000
29	60.7	2,785,000,000
30	107.1	2,060,000,000

19 days. The total count was obtained on a medium containing milk powder and tomato juice on which both cultures grew readily and the *Esch. coli* count on an ammonium phosphate agar on which *Str. lactis* did not grow. The lactic colonies were obtained by difference. The synthetic medium did not support

an active growth of the *Esch. coli* culture and it is possible that the count obtained was below the number actually present. The discrepancy between the coli counts in tables 2 and 3 may be due to this factor rather than to the inhibiting effect of the lactics.

There was evident at the beginning an inhibition of the *Str. lactis* but in 8 days a normal population had been reached.

TABLE 3

Esch. coli and Str. lactis in continuous flow of low sugar broth

AGE	FLOW OF BROTH PER CENT OF CAPACITY OF FLASK	COLI PER CUBIC CENTIMETER	LACTIC PER CUBIC CENTIMETER
days			
1	42.8	267,000,000	245,000,000
2	71.4	436,000,000	274,000,000
3	71.4	2,330,000,000	195,000,000
4	57.1	3,060,000,000	770,000,000
5	57.1	3,525,000,000	470,000,000
6	50.0	2,550,000,000	725,000,000
7	96.4	2,965,000,000	700,000,000
8	125.0	1,455,000,000	2,000,000,000
9	107.1	1,045,000,000	2,125,000,000
10	128.5	1,750,000,000	1,640,000,000
11	92.8	1,880,000,000	1,555,000,000
12	196.4	1,740,000,000	1,460,000,000
13	125.0	655,000,000	2,020,000,000
14	85.7	850,000,000	2,500,000,000
15	192.8	2,720,000,000	1,270,000,000
16	114.2	1,480,000,000	2,250,000,000
17	135.7	980,000,000	900,000,000
18	121.4	146,000,000	714,000,000
19	117.8	173,000,000	717,000,000

In the latter part of the experiment there was a distinct falling off in the number of *Esch. coli* but, in refilling, the culture flask was contaminated and the experiment terminated before definite results were obtained.

In all of these experiments the population level was maintained in spite of the heavy emigration of cells from the culture. If the cells were evenly distributed throughout the medium it would be necessary for the culture to reproduce itself completely about once every 30 hours, and in some cases even more frequently. In

this respect the conditions were not exactly comparable to those in a colony of multicellular organisms and another experiment was devised which, in some measure at least, overcame this objection.

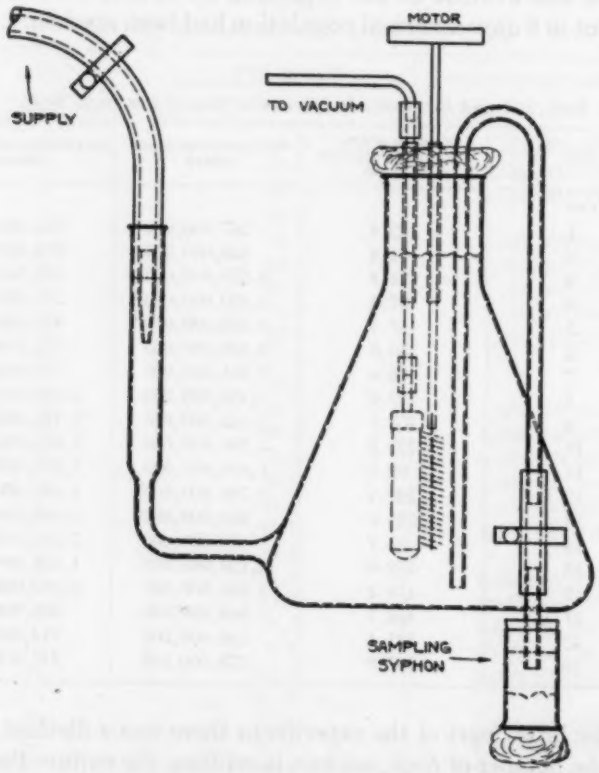

FIG. 2. FLASK ARRANGED TO REMOVE OVERFLOW THROUGH FILTER

In this experiment the broth flowed into the culture flask (fig. 2) as usual but the overflow was carried off through a Berkefeld filter suspended in the culture. The accumulation of cells on the surface of the filter was reduced to a minimum by a revolving brush. The brush rotated constantly but it was neces-

sary to turn the filter manually at frequent intervals to expose the entire surface to the action of the brush. When the filter was removed at the end of the experiment it was free of sediment except on the end where the brush did not reach. The flow of broth into the culture was regulated to balance as closely as possible the outflow through the filter. The greater part of the time about one-third of the filter was submerged and there was no great variation in the volume of the culture.

The rate of flow was sufficient to change the broth in the culture flask from once to over five times every 24 hours. An active culture of *Str. lactis* was used. The results are given in table 4.

TABLE 4

The growth of Str. lactis in a continuous flow with surplus removed by filtration

AGE FROM INOCULATION	OVERFLOW PER CENT OF CAPACITY OF FLASK	BACTERIA PER CUBIC CENTIMETER
hours		
24	185	
41	414	
65	557	243,000,000
89	428	355,800,000
113	300	291,500,000
140	328	291,800,000
159	214	368,600,000
166	100	
183	0	404,200,000
207	0	278,500,000
231	0	48,000,000
255	0	21,250,000

Notwithstanding the abundant supply of nutritive material and the prompt removal of filterable products the population maintained itself at a nearly constant level for 7 days. The comparatively low number maintained in this experiment is probably normal for the culture used, which was not identical with the culture used in the earlier experiments.

Since there was no migration of cells from the culture in this experiment, the only multiplication that could have taken place was that necessary to balance the death rate. If growth continued at the normal rate there must be a correspondingly rapid death rate to hold the population at a nearly constant level.

If the cells did not disintegrate rapidly there would be an accumulation of dead cells and a microscopic count should show a much higher number than the plate count. A microscopic count made at 113 hours was 2,680,000,000 or about ten times the plate count. Since this culture had a decided tendency to form short chains the difference observed was only what would be expected.

It would be difficult to determine positively whether the cells do or do not disintegrate rapidly after death. In an earlier paper[1] it was shown that in a lactic culture in which multiplication had ceased and the number of viable cells was so reduced that 1/10,000 cc. was required to produce growth in milk the direct count was approximately the same as when the culture was at its maximum. This would indicate that the disintegration of the cells was slow.

When the flow of broth was stopped in the flask with the filter, three days elapsed before there was a material decrease in the count. This, we think, indicates that the cells were not dying so long as the flow continued.

It appears from these experiments that, if the conditions are comparable, bacteria will follow the laws governing the population of a colony of multicellular organisms and will maintain a constant population indefinitely. This, however, does not explain why, with an abundant food supply and with filterable products of growth removed, the population should rise rapidly to a fixed number and remain constant at that level.

It is probable that our analogy should be drawn, not between colonies of bacteria and multicellular organisms but between bacteria and an individual multicellular organism.

The normal size of an animal, a tree, or an insect is fixed by heredity. Neither favorable environment nor nutrition will cause an animal or a plant to develop beyond certain limits which are fixed for each species. Even the parts of an individual are constant for the species or strain.

Bacterial colonies on solid media show the effects of a similar law. The typical streptococcus colony on agar is invariably a

[1] Rogers, L. A. and Whittier, E. O. Limiting factors in the lactic fermentation. Jour. Bact., 16, 211–229 (1928).

small sphere, and while the size may be increased somewhat by providing an especially favorable medium, nothing will make it differ materially from its ancestor colonies. In liquid media the maximum population is fairly constant at a number which seems to be fixed for each species or strain.

An analogy of this nature serves a useful purpose as a means of illustrating an obscure phenomenon but should be accepted with caution. The fact that different types of organisms follow the same general course of growth and maintenance does not necessarily prove that they are governed by identical laws. In any event the consideration of heredity as a factor does not really explain why growth ceases when the colony has reached a certain size or the population has attained a certain density.

It is difficult to conceive of the control of a phenomenon of this nature which does not have its basis in some physico-chemical balance. Heredity can only determine the point at which this balance becomes effective in checking growth.

There is a possible application of these results to the fermentation industry. In the usual intermittent method a large part of the time is used in sterilizing equipment and medium, developing the culture to its maximum strength and, when fermentation is completed, emptying the tanks and preparing them for another run. If a fermenting vat could be supplied continually with fresh fermentable material and the surplus removed, it should be possible to maintain the fermentation at its maximum speed for an extended period. We have been able to maintain a lactic fermentation under laboratory conditions for an indefinite time. In these experiments we have used a mixed culture containing a lactobacillus and a mycoderm. This combination grows vigorously and produces lactic acid.

The culture flask, filled to the overflow level, held about 800 cc. and was maintained at 35°C. A heavy suspension of sterilized calcium carbonate was run into the culture at intervals and kept in suspension by a stirrer. The fermenting material was an infusion broth containing tomato juice and 5.92 per cent lactose. The experiment, the results of which are shown in table 5, was continued for 20 days. On the second day after inoculation the

flow of broth was started slowly and 140 cc. overflow obtained on the morning of the third day.

On account of the difficulty in adjusting the flow accurately there was a variation in the overflow from 140 to 900 cc. per day. This caused a variation in the total amount of sugar fermented and the rate per hour. The maximum fermentation was obtained in the period in which 900 cc. flowed through the flask. In this

TABLE 5

The continuous lactic fermentation of broth containing 5.92 per cent lactose

AGE	OVERFLOW	SUGAR FERMENTED			LACTIC ACID PER CENT OF TOTAL SUGAR FERMENTED
		Grams	Per cent of total in overflow	Grams per hour	
days	*cc.*				
3	140	8.08	97.4	0.33	
4	575	34.04	97.4	1.41	
5	670	39.06	98.4	1.62	
6	900	52.65	98.8	2.19	
7	750	41.85	94.2	1.74	
8	400	18.76	79.2	0.78	57.9
9	560	28.73	86.6	1.19	
10	500	23.50	79.3	0.97	
11	480	23.18	81.5	0.96	
12	500	26.45	89.3	1.10	75.8
13	460	25.30	92.9	1.05	75.3
14	460	26.27	96.4	1.09	
15	340	19.52	96.9	0.81	
16	450	26.24	98.4	1.09	
17	340	19.28	85.7	0.80	
18	230	13.09	96.1	0.54	
19	630	35.15	94.2	1.46	68.8
20	575	27.37	80.4	1.14	89.1

period 52 grams of sugar were fermented at the rate of 2.19 grams per hour. Some undetermined factor affected the efficiency of the fermentation as shown by the percentage of sugar fermented. This varied from 77 to 98.8 per cent. In other words, on one day the broth came through the culture flask with 25 per cent of the sugar unfermented. This variation was independent of the rate of flow. It is possible that it may have been caused by failure to keep the reaction of the culture within the limits of greatest activity.

The efficiency of the fermentation in producing lactic acid is shown by the sixth column of table 5. This shows the percentage of the total sugar fermented which was converted to lactic acid. In other words it is lactic acid expressed as percentage of the theoretical yield.

SUMMARY

Cultures of *Str. lactis* or *Esch. coli*, in flasks through which broth was flowed slowly, maintained a constant population level as long as the experiment was continued.

Under similar conditions *Str. lactis* and *Esch. coli* grew together, each one maintaining a high population. At the beginning, there was an apparent inhibition of *Str. lactis* and, later, a distinct falling off in the number of *Esch. coli*.

In a culture flask with a continuous flow of fresh broth into the flask, and the overflow removed by filtration so that there was no emigration of cells out of the flask, *Str. lactis* maintained a constant population level.

Under laboratory conditions a continuous lactic fermentation could be maintained indefinitely by providing a continuous supply of sterile lactose broth. Under favorable conditions an 800-cc. culture delivered every 24 hours from 500 to 900 cc. of effluent from which practically all of the sugar had been fermented.

IS THE EIJKMAN TEST AN AID IN THE DETECTION OF FECAL POLLUTION OF WATER?

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INTRODUCTION

The present standards of the American Public Health Association (1925) have designated Gram-negative non-spore-forming aerobic rods which ferment lactose with the production of gas as the organisms to be searched for to detect fecal pollution. From time to time other organisms have been proposed wholly or partially to replace these bacteria, but, as stated so definitely and convincingly by Levine (1921), *Bacterium coli* is the organism which best fulfills the requirements for an index of fecal pollution. It is constantly present in the feces of warm blooded animals, is seldom obtained except from feces or from substances recently polluted, is slightly more resistant than intestinal bacterial pathogens, but is not sufficiently resistant to remain long in water after the water has been purified enough to kill pathogens.

However, as shown by many studies summarized by Levine and since added to by others, the related organism, *Bacterium aerogenes*, included in the above description, is in no way an index of fecal pollution. Neither *B. aerogenes* nor its very close relative, *Bacterium cloacae*, is present in feces in very large numbers. They are present in fairly large numbers in soil, plant residues and other places not associated with feces, and are more resistant to storage than *B. coli*. *B. aerogenes* is undoubtedly a separate species, differing from *B. coli* in several correlating characters, namely the production of acetyl-methyl carbinol (Voges-Proskauer test), the production of a higher CO_2/H_2 ratio, the utilization of uric

acid as a sole source of nitrogen, and the production of a higher pH in the methyl-red test. Although the American Public Health Association standards do not distinguish between *B. coli* and *B. aerogenes* in the "completed test," there can be little doubt that *B. aerogenes* has no value as an index of fecal pollution. It can be fairly readily distinguished from *B. coli* on the eosine-methylene blue (Levine (1918, 1921)) or the triple dye agar plates (Skinner and Murray (1924)), as well as by the more accurate methods summarized by Standard Methods (1925).

B. coli itself, however, is occasionally isolated from soil, is often found in feces of cold blooded animals, and occasionally occurs in streams free from apparent human pollution. Koser (1924) has found that, in general, only such strains as are found in soils are able to utilize citrates as a sole source of energy. Since all strains of *B. aerogenes* also utilize citrates, this medium offers an interesting possibility for the elimination of all members of the coli-aerogenes group which are of no sanitary importance. Although as yet enough work has not been done with strains of *B. coli* isolated from feces to prove absolutely the importance of Koser's findings, it is a distinct probability that only the citrate negative organisms should be considered in water analysis. The results of Pawan (1926) who found that citrate negative strains were present in 98 per cent of the samples of cow and human feces and citrate positive strains in 100 per cent of the samples of unpolluted water, certainly confirm Koser's results, although it is probable that most of his non-fecal organisms were *B. aerogenes*. Other workers have obtained somewhat similar results.

Eijkman (1904) has suggested a means of separating the *B. coli* originating in the feces of warm blooded animals from the strains characteristic of cold blooded animals. This consists in incubating inoculated glucose-peptone broth at 46°. Gas formation is said to indicate the presence of *B. coli* from warm blooded animals. *B. coli* from cold blooded animals, practically all *B. aerogenes*, (and *B. cloacae*), *Bacterium proteus*, and the various fermenting anaerobic rods are said to be completely inhibited as far as gas production is concerned. Although of great importance, (if the above is true) this test has not, so far as

we are aware, been used at all in North America except in a limited way by Skinner and Murray (1926) and especially by Leiter (1929) who made a fairly intensive study of it.

However, even if these organisms of little importance, giving presumptive tests, be eliminated, not a great deal of work has been done to show that all or nearly all of the *B. coli* from feces of warm blooded animal origin ferment glucose at 46°. Leiter isolated 143 strains of *B. coli* from human feces all of which produced gas 46°, although five at times produced less than 0.5 cc. in the Smith fermentation tube. Similar results were obtained by Salus and Hirn (1923). Positive Eijkman tests have rarely been encountered from water of known high sanitary quality by any of the European workers. Most of the work done on the test, which has been studied rather intensively in Europe and summarized by Leiter (1929) and by Barth (1930), indicates that false positive tests are rarer than in the case of the usual methods with lactose broth at 37.5°.

It was decided that further work was necessary to indicate whether or not false negative tests could be expected, that is, if all or nearly all of the *B. coli* from the feces of humans are able to produce gas at 46° from glucose broth. If so, since false positives are said to be rare, it should offer a relatively cheap and simple means of detecting fecal pollution.

First of all, if all the *B. coli* grow at 46°, their numbers in feces should be as large when determined by the Eijkman media at 46° as by the lactose broth at 37.5°. Fortunately, the dilution method offers us a rather accurate means of determining numbers (McCrady (1918), Buchanan and Fulmer (1928)). It was also deemed advisable to determine whether the gas production was due to *B. coli*, *B. aerogenes*, or lactose fermenting anaerobes, although evidence is not convincing that any large numbers of *B. aerogenes* are present in feces.

EXPERIMENTAL

Thirty-seven samples of feces from hospital patients and from persons in normal health were diluted in three or four decimal dilutions, 1 to 10^6 to 1 to 10^9 inclusive, in replicates of five

Durham fermentation tubes. Both the glucose broth (Difco peptone 12.5 grams, glucose 12.5 grams, NaCl 6.25 grams per liter) and lactose broth (peptone 10 grams, lactose 10 grams, beef extract 3 grams, NaCl 5 grams per liter, pH 7.2) were inoculated from the same dilutions, at the same time and with the same pipettes. The glucose medium is the same as that used by Leiter (1929) and is of the same concentration as Eijkman's after being diluted with water. After incubating the lactose broth at 37.5° and the glucose media at exactly 46° in a Central Scientific Company water bath with a DeKhotinsky thermoregulator for twenty-four hours, the tubes with any gas at all were streaked on standard eosine-methylene blue agar plates. Those showing gas in forty-eight hours were streaked the following day. Since the streak plates were all confirmed, and since all plates contained only typical *B. coli* colonies as evidenced by the greenish metallic sheen, the confirmation was discontinued, after 157 streak plates had been made. Like Leiter, we found that the streak plates from lactose broth inoculated with feces showed pure or almost pure cultures of *B. coli*. That they were citrate negative, we cannot say, but at any rate they were obviously fecal. Not a single plate of the 157 cultures streaked from either glucose or lactose broth cultures of feces showed a colony resembling *B. aerogenes* or *B. cloacae*.

These results (table 1), calculated from the tables of McCrady (1918) (Buchanan and Fulmer (1928)), show that not all of the lactose fermenting organisms (exclusively *B. coli* by eosine-methylene blue agar plate appearance) produce gas in Eijkman's broth in forty-eight hours. They further show that twenty-four hours at 46° is not a sufficient time for many cultures. Some cultures have large amounts of gas in twenty-four hours, others show none at all at this period but develop gas in forty-eight hours. On the other hand, it was found that those cultures which produced gas in twenty-four hours contained few or no living organisms in forty-eight hours, the acidity apparently being more toxic at this high temperature. All plates streaked from the Eijkman broth contained fewer colonies than those streaked from the lactose broth. All the lactose broth tubes

TABLE 1

Comparison of lactose broth at 37.5° and glucose broth at 46° (Eijkman method) for detecting B. coli in feces

DATE	LACTOSE BROTH 37.5°		GLUCOSE BROTH 46°			RATIO OF NUMBER OF B. COLI, LACTOSE BROTH METHOD TO NUMBER EIJKMAN METHOD
	Tubes showing gas in dilutions of 1:10², 1:10³ and 1:10⁴, 48 hours	Most probable number *B. coli* as shown by gas formation	Tubes showing gas in dilutions of 1:10², 1:10³ and 1:10⁴, 24 hours	Tubes showing gas in dilutions of 1:10², 1:10³ and 1:10⁴, 48 hours	Most probable number *B. coli* as shown by gas formation	
		millions per gram			*millions per gram*	
December 20, 1929	5-4-1	17.0	1-0-0	3-1-0	1.1	15.5
	5-5-1	35.0	0-0-0	0-0-0	<0.2	175.0†
	5-0-0	2.5	3-0-0	3-1-0	1.1	2.3
December 21, 1929	5-5-1	35.0	4-4-0	5-5-0	25.0	1.4
	5-5-5	>180.0	3-3-1	5-5-3	90.0	2.0
	5-5-5	>180.0	0-0-0	0-0-0	<0.2	900.0†
	5-5-5	>180.0	5-5-1	5-5-2	35.0	5.1
	5-4-0	13.0	5-2-0	5-4-0	13.0	1.0
	5-2-0	5.0	4-1-0	5-2-0	5.0	1.0
December 24, 1929	5-5-3	90.0	Not counted	4-0-0	1.3	69.2
	5-5-5	>180.0	Not counted	5-5-3	90.0	2.0
	5-5-5	>180.0	Not counted	5-5-4	160.0	1.1
	5-5-5	>180.0	Not counted	5-5-4	160.0	1.1
	5-5-5	>180.0	Not counted	5-4-2	25.0	7.2
December 31, 1929	3-0-1*	11.0	0-0-0*	0-0-0*	<2.0	5.5
	1-0-0*	2.0	0-0-0*	1-0-0*	2.0	1.0
January 2, 1930	5-5-4	160.0	0-0-0	3-0-1	1.1	145.5†
	5-5-1*	350.0	0-0-0	3-1-0	1.1	318.2†
	5-5-1*	350.0	4-2-0*	4-2-0*	20.0	17.5
	2-2-0	0.9	1-0-0	1-0-0	0.2	4.5
January 3, 1930	5-3-1	11.0	5-2-0	5-4-0	13.0	0.8
	5-5-1	35.0	5-0-0	5-5-1	35.0	1.0
	1-0-0	0.2	0-0-0	0-0-0	<0.2	1.0
	5-5-3*	900.0	5-2-0	5-5-0	25.0	36.0
January 4, 1930	4-0-0	1.3	0-0-0	0-0-0	<0.2	6.5
	4-2-0	2.0	0-0-0	3-1-0	1.1	1.8
	5-4-0*	130.0	0-0-0	3-1-0	1.1	118.2†
	2-0-0	0.5	2-0-0	2-0-0	0.5	1.0

TABLE 1—*Concluded*

DATE	LACTOSE BROTH 37.5°		GLUCOSE BROTH 46°			RATIO OF NUMBER OF B. COLI, LACTOSE BROTH METHOD TO NUMBER EIJKMAN METHOD
	Tubes showing gas in dilutions of $1:10^6$, $1:10^7$ and $1:10^8$. 48 hours	Most probable number *B. coli* as shown by gas formation	Tubes showing gas in dilutions of $1:10^6$, $1:10^7$ and $1:10^8$. 24 hours	Tubes showing gas in dilutions of $1:10^6$, $1:10^7$ and $1:10^8$. 48 hours	Most probable number *B. coli* as shown by gas formation	
		millions per gram			*millions per gram*	
January 15, 1930....	5-5-1*	350.0	4-4-0*	5-4-3*	300.0	1.2
	4-1-0	1.7	0-0-0	0-0-0	<0.2	8.5
	2-0-0	0.5	0-0-0	0-0-0	<0.2	2.5
	2-0-0	0.5	0-0-0	0-0-0	<0.2	2.5
	5-3-0	8.0	0-0-0	0-0-0	<0.2	40.0
January 18, 1930....	5-5-3	90.0	0-0-0	0-0-0	<0.2	450.0†
	5-4-0*	130.0	2-0-0	3-2-0	1.4	92.9
	5-3-1*	110.0	0-0-0	0-0-0	<0.2	550.0†
	5-5-1*	350.0	0-0-0	2-0-0	0.5	700.0†
						11.5 mean
						5.1 median

* Tubes $1:10^7$, $1:10^8$, $1:10^9$ dilutions.

† Values not counted in determining mean.

were either positive in twenty-four hours or negative in forty-eight hours. The table indicates that the Eijkman test should be expected to give many false negatives. Furthermore, it was discovered that the organisms were quickly killed after gas formation had taken place, which probably also explains Leiter's failure to confirm all his tests. Whether the failure to grow at 46° was due to strain variation or to relatively greater resistance to the high temperatures by some of the organisms at certain physiological ages we cannot say. These conclusions were based upon more than one thousand inoculations.

Similar tests were made also with water. For this purpose water was used from the Mississippi river, above the city of Minneapolis, as it comes into the filtration plant.[1] Three decimal

[1] We are indebted to Mr. I. A. Montank, bacteriologist at the Minneapolis filtration plant, who on several occasions took the samples and brought them to us.

dilutions from 1 cc. to 0.01 cc. were used. It was evident at the first preliminary trial that standard eosine-methylene blue agar could not be used for confirmation tests, since non-lactose fermenters in very many cases prevented isolation and identification of the coli-aerogenes organisms. The addition of 1:100,000 crystal violet was found largely to prevent this, as had previously been found to be the case (Skinner and Murray (1924)). Very possibly the use of crystal violet (Hall and Ellefson (1918)), or other dyes, or NaOH (Janzig and Montank (1928)) in the lactose broth would eliminate some of the unconfirmed tests. However, in our case it was not the lactose fermenting anaerobes which gave the false positive presumptive tests so much as the overgrowth of non-lactose fermenting organisms on the plate, which was prevented by crystal violet. There is ample evidence in the literature that such a concentration of the dye is in far too great dilution to cause marked inhibition of *B. coli*.

After incubation for twenty-four hours, the tubes showing gas were streaked on the triple dye agar. From those developing gas in the 24-to-48-hour period, streaks were made after forty-eight hours incubation. Colonies were picked, one colony most resembling *B. coli*, from each plate, and planted on agar slants. These were then inoculated into lactose broth for confirmation, peptone glucose broth for Voges-Proskauer, peptone broth for indol, and Simmon's (1926) agar for the detection of citrate utilization. In case the cultures showed acid and gas from lactose broth, were negative to the Voges-Proskauer test and did not utilize citrates in 48 hours, they were recorded as *B. coli*. If they showed opposite reactions in the tests, save acid and gas on the lactose broth, they were called *B. aerogenes*. If no gas resulted from lactose broth, they were recorded as not confirmed, as were also plates on which no growth resulted after streaking. All other cultures were reinoculated into lactose broth, reisolated and again planted on the media. In case they checked with the first reading, they were recorded as such, or if *B. coli* or *B. aerogenes* reactions appeared after repurification (as was frequently the case) these were duly recorded in the table. Table 2 shows the results.

The table shows many "skips" due most likely to overgrowth of

TABLE 2

Comparison of lactose broth at 37.5° and glucose broth at 46° (Eijkman method) in their effectiveness in detecting typical B. coli and in repressing B. aerogenes and citrate-utilizing B. coli in water

SAMPLE NUMBER	LACTOSE BROTH 37.5°			GLUCOSE BROTH 46°			RATIO OF NUMBER OF TYPICAL B. COLI LACTOSE BROTH AT 37.5° COMPARED TO NUMBER EIJKMAN METHOD
	Tubes containing *B. coli* inoculated with 1, 0.1 and 0.01 cc. water	Most probable number *B. coli* per 10 cc. water	Other members of colon group than typical *B. coli* isolated	Tubes containing *B. coli* inoculated with 1, 0.1 and 0.01 cc. water	Most probable number *B. coli* per 10 cc. water	Other members of colon group than typical *B. coli* isolated	
1	4-0-0	13	1 *B. aerogenes*	1-0-0	2		6.5
2	3-0-0	8		0-0-0	<2		4.0
3	2-2-0	9	3 *B. aerogenes*	3-0-0	8		1.1
4	4-1-0	17	1 not confirmed	4-1-0	17		1.0
5	3-0-0	8	1 *B. aerogenes*	2-0-0	5		1.6
6	2-2-0	9	4 *B. aerogenes* 1 not confirmed	2-1-0	7	2 *B. aerogenes* 1 not confirmed	1.3
7	3-3-0	17	2 *B. aerogenes*	2-0-0	5	2 *B. aerogenes*	3.4
8	3-2-0	14	2 *B. aerogenes*	0-0-0	<2		7.0
9	4-5-1	50	1 *B. aerogenes*	4-2-0	20		2.5
10	2-3-0	12	1 citrate + *B. coli* 5 *B. aerogenes*	2-1-0	7	1 *B. aerogenes* 1 not confirmed	1.7
11	2-2-0	9	3 citrate + *B. coli* 3 *B. aerogenes*	1-1-0	4	1 *citrate* + *B. coli* 2 *B. aerogenes*	2.3
12	3-3-1	20	3 *B. aerogenes*	1-1-0	4	4 *B. aerogenes* 1 not confirmed	5.0
13	3-4-0	20	2 *B. aerogenes* 1 not confirmed	1-1-0	4	1 citrate + *B. coli* 1 *B. aerogenes* 2 not confirmed	5.0
14	2-1-0	7	2 *B. aerogenes*	0-0-0	<2	1 citrate + *B. coli* 3 *B. aerogenes*	3.5
15	2-3-0	12	5 *B. aerogenes*	2-1-0	7	2 *B. aerogenes*	1.7
16	4-3-0	25	1 citrate + *B. coli* 1 not confirmed	5-0-0	25	1 *B. aerogenes*	1.0
17	3-2-0	14	3 *B. aerogenes*	2-0-0	5	5 not confirmed	2.8
18	2-0-0	5	2 *B. aerogenes* 1 not confirmed	2-0-0	5		1.0
19	4-3-1	30	1 citrate + *B. coli*	2-0-0	5	2 *B. aerogenes* 1 not confirmed	6.0
20	3-3-0	17	3 *B. aerogenes* 1 not confirmed	3-0-0	8	2 *B. aerogenes* 1 not confirmed	2.1
	103 total			48 total			2.4 mean 3.0 median

B. aerogenes and other bacteria. For this reason the results should not have as much weight as those of table 1. They seem to indicate, however, that the Eijkman test fails to detect all the fecal *B. coli*. The large number of unconfirmed tests from the Eijkman media probably indicate cultures in which *B. coli* had been present but had died, due to acidity at the high temperature. Counting all these "not confirmed" as "fecal *B. coli*," table 3 shows that even so, all the *B. coli* are not always obtained by the Eijkman method. It further shows that a fair number of *B. aerogenes* and atypical

TABLE 3

The types of members of the coli-aerogenes group recovered from the inoculation of the same number of lactose broth tubes at 37.5° and of glucose broth at 46° with water from a polluted stream

TYPE RECOVERED	LACTOSE, 37.5°		GLUCOSE, 46°	
	Number recovered	Per cent	Number recovered	Per cent
B. coli; non-spore-forming rods, acid and gas in lactose broth, Voges-Proskauer −, no utilization of citrates	103	64.8	48	70.6
B. aerogenes; as above except Voges-Proskauer + and utilization of citrates	43	27.0	22	25.9
B. coli, atypical; like *B. coli* above except utilization of citrates	7	4.4	3	3.5
Not confirmed; gas formation in tubes, but lactose fermenting aerobes not isolated	6	3.8	12	—*
Total	159		85	

* Not confirmed included in percentage of *B. coli*. See text.

B. coli (citrate positive, or "non fecal" according to Koser) are apt to be involved in a positive Eijkman test. Thus, we were not able to confirm the work of de Graaff (1922) who found that Voges-Proskauer positive, methyl red negative strains of the colon bacilli (*B. aerogenes*) were repressed in the Eijkman test.

DISCUSSION

Our results indicate that, even if the production of gas at 46° from glucose broth be a fair index of pollution from feces, the lack

of such gas formation does not prove the absence of pollution. The absence of a positive presumptive lactose broth test at 37.5° of course indicates the lack of fecal pollution. Together, the tests may be of benefit. Alone, a positive lactose broth presumptive test needs confirmation and differentiation of *B. coli* from *B. aerogenes* or, probably better still, differentiation of citrate from non-citrate utilizing organisms. Alone, a negative Eijkman test does not appear to the authors to be of much value. The results obtained by us indicate that twenty-four hours is not enough time for the Eijkman test. The fact that *B. aerogenes* is seldom encountered in feces and is, therefore, of no significance in water analysis except in so far as it leads to confusion, has been confirmed. The addition of 1:100,000 crystal violet to the standard eosine-methylene blue agar has made the confirmation of presumptive tests easier and more certain.

The fact that our results are on the whole unfavorable to the Eijkman test detracts in no way from Leiter's work. His results were on isolated strains. Ours were quantitative and on feces. Also, his results, as well as those of others, show conclusively that the Eijkman test does eliminate a great many of the positive tests, the origin of which was cold blooded animals. Our results are more in agreement, however, with those of Hehewerth (1912) who found that only 38.8 per cent of the 36 strains isolated from feces gave an Eijkman reaction, and with those of Barth (1930) who found that only 37 per cent of the 31 strains isolated from human feces grew at 45°. This latter work appeared after our experimental data had been compiled and while the manuscript was being written. In all of the work on the test previous to ours, as far as we are aware, no worker has utilized to any great extent the dilution method. This method seems to us to give the most pertinent results, for unless all *B. coli* from feces will grow at 46° in glucose broth (and our results show they do not) and will grow whether 1 or 100 cells are used as an inoculum, the Eijkman test is of little value in detecting all waters containing *B. coli*, and especially is it without value in obtaining quantitative data as to the amount of pollution.

SUMMARY

1. Only a small percentage of the *B. coli* from human feces produced gas in forty-eight hours in glucose-peptone broth at 46° (Eijkman test).

2. Many strains produced gas in twenty-four hours in the Eijkman test, but forty-eight hours were necessary to include all the gas formers.

3. Many "typical" *B. coli* from water from a polluted steam failed to grow in the Eijkman broth at 46°.

4. The Eijkman test did not eliminate all positive tests due to *B. aerogenes* and citrate positive *B. coli*.

5. Only *B. coli* (never *B. aerogenes*) was found in human feces.

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THE BACTERIOPHAGE CONTENT OF SEWAGE AND ITS ACTION UPON BACTERIAL ORGANISMS

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It has long been recognized that sewage tends to purify itself of its primitive bacterial content. This activity has been ascribed to many different factors and it may be deemed certain that the picture of the changes resulting in lowered bacterial count of sewage when definitely known will be found to be complex. It is the purpose of this discussion to add still another agency to the list of those already identified with production of changes in the bacterial content of crude sewage.

A normal habitat of bacteriophage is within the intestinal content of higher animals. Since bacteriophage is found ordinarily within the intestinal tract, it is present likewise in feces and, logically therefore, in sewage. It is not our desire at this time to discuss the nature of bacteriophage activity for it makes very little difference whether this factor is to be classed as filterable virus or as an enzyme since its power as a lytic agent is accepted. Moreover, its increase in potency by repeated contact with organisms in the logarithmic stage of multiplication has been thoroughly substantiated. Bacteriophage has a certain range of adaptability from strain to strain of bacterial microörganisms although the extent of our present field of knowledge is not sufficient to describe accurately what these limitations are. The adaptability of sewage bacteriophage to a series of bacterial forms has been demonstrated repeatedly and indeed we have utilized it upon numerous occasions for elaboration of preparations to be utilized for treatment of infections of the genitourinary tract. Similar observations have been published also by Caldwell (1928). In addition, there are very many strains of

bacteriophage in sewage since the content of fecal material is derived from many different individuals, both man and higher animals. The degree of activity of phage against various bacterial forms as well as their "specificity" varies with different persons. Therefore, sewage should contain a great variety of strains, active against many different types of intestinal bacterial organisms.

However, there are certain factors which may be expected to limit rather sharply the possible influence of bacteriophage as a purifying agent in sewage. Multiplication of the original intestinal organisms in the sewage takes place much more slowly than within the animal body for many of these organisms are not in the logarithmic period of growth. Bacteriophage is most effective when propagated in contact with young, rapidly multiplying organisms as has been stated by d'Herelle (1926). The maximum effectiveness of the various strains of phage in sewage therefore is not to be expected although the presence of many different bacteriophages in the sewage is certain. In addition, it has been shown by Marshall (1925) that the action of bacteriophage in its multiplication within or upon young bacterial organisms is a function of the numbers of the bacteria available for the purpose. The smaller the number of bacteria in a suspension even though they be at the optimum stage of growth, the less the likelihood of contact between bacteriophage particles and their food material, which in this instance is the bacteria. Temperature relationships in sewage, also, are not optimum for the proliferation of phages. Thus, within the medium, conditions are not the best possible for growth and therefore effectiveness of bacteriophages. Nevertheless, there should be a considerable activity on the part of the phages against sewage organisms present within the sewage even though conditions are not optimum. Any degree of bacteriophage lysis within sewage will act as an agency to diminish bacterial content. Activity on the part of the bacteriophage content of sewage against its contained organisms, we have endeavored to demonstrate.

Our method of procedure has been the following. Sewage, either entirely untreated or taken at various stages of the process

of purification, has been collected in sterile bottles. These samples have been plated out on lactose litmus agar in order that the various forms present might be examined as to colony formation and ability to split the sugar. After twenty-four hours incubation at 37° a series of acid producers and a second series of those which did not produce red colonies were transferred to beef agar slants. No further attempt was made to classify these cultures. A portion of the sewage sample to be examined for the presence of bacteriophage was filtered, first through ordinary

TABLE 1

Lytic action of Sonoma, California, sewage upon organisms isolated from that sewage, with twenty-four hours incubation at 30°C.

CULTURE	EFFLUENT SEPTIC TANK	SEWAGE SETTLING POND	SEWAGE SETTLING POND
A1	−	−	−
A2	−	−	−
A3	++	++	+++
A4	++	−	++
A5	+	+	+
A6	+	+	−
A7	+++	+++	−
A8	−	++	−
B1	+++	+++	+++
B2	++++	+++	+++
B3	+++	+++	+++
B4	+++	+++	+++
B5	++++	++++	++++

− = no inhibition; + = very slight lysis as indicated by zones of clarity or slightly decreased turbidity; ++ = more evident inhibition; +++ = moderate lysis; ++++ = complete lysis.

filter paper to remove gross particles, and then through a Berkfeld "N" candle. Thus from the sample under test we obtained, first, a series of bacterial organsms in pure culture and second, a sterile filtrate.

We then carried through tests to determine the presence of bacteriophage in the sewage. The various organisms were inoculated into beef infusion broth tubes containing approximately 10 cc. of the medium. Two tubes were prepared for each culture. Of the two tubes thus inoculated, one was then treated by addi-

tion to it of 1 cc. of the filtered sewage. The second tube, to which no addition was made, served as a control. Incubation then proceeded at 30°. This tempeature we have utilized in our work since it induces somewhat slower growth and, therefore a longer period is offered for maximum activity of bacteriophage upon the multiplying bacteria. Following twenty-four hours incubation, these tubes were then observed in pairs for contrast in turbidity between the culture treated with sewage and its control. Lysis was demonstrated by degrees of clarity of the treated culture in comparison to the one not treated.

Experiment I. Sonoma, California, Sewage Purification System. In this plant the sewage first passes through a septic tank and then through a series of ponds where a layer a few inches deep is exposed to air oxidation. Three specimens were utilized for this test: (1) Effluent from septic tank. (2) and (3) Fluids from two of the settling ponds. The filtrate of each sample was brought into contact with organisms derived originally from that particular specimen. As will be noted below in table 1, eight acid producers and five non-acid producers upon lactose litmus agar had been isolated from each sample.

It thus becomes very evident that sewage filtrate inhibits in marked fashion the growth of certain organisms derived from that sewage when conditions are made optimum for growth. For some unexplained reason, inhibition is much more marked upon forms inactive in lactose than upon the lactose splitters.

Experiment II. Effect of Sonoma, California, sewage upon organisms derived from the septic tank of the system. These samples were taken approximately two and one-half months later than those noted in the previous experiment. It was now at the end of the rainy season. Much storm water had been flushing through the system and it was desired to learn whether the lytic action at this plant had become attenuated by such flooding. Two samples of sewage were taken: (1) The septic tank effluent. (2) The effluent just below the first of the subsequent oxidizing ponds. The organisms used here were isolated from the sewage of the septic tank only. Freshly inoculated cultures of these were in turn placed in contact with 1 cc. amounts of filtered sew-

age from each sample. It will be noted that nine acid producers and five non-lactose splitters have been incorporated within this series. Results are given in table 2.

It is thus demonstrated that the lytic activity of this sewage following long continued and heavy rainfall had become diminished markedly. Nevertheless, distinct activity was apparent upon those forms which were inactive with lactose.

Experiment III. Near Sonoma, California, there is also a State institution for women. This so called "Sonoma Home" has its own separate sewage disposal plant consisting of a septic

TABLE 2

Lytic action of Sonoma, California, sewage upon organisms isolated from that sewage with twenty-four hours incubation at 30°C.

Samples taken following heavy flushing of system by flood waters

CULTURE	SEPTIC TANK EFFLUENT	SETTLING POND FILTRATE	CULTURE	SEPTIC TANK EFFLUENT	SETTLING POND FILTRATE
A1	−	−	B1	++++	++
A2	−	−	B2	−	−
A3	−	−	B3	++	−
A4	−	−	B4	+	+
A5	−	−	B5	Lost	+
A6	−	−			
A7	+	+			
A8	−	−			
A9	−	−			

− = no inhibition; + = very slight lysis as indicated by zones of clarity or slightly decreased turbidity; ++ = more evident inhibition; +++ = moderate lysis; ++++ = complete lysis.

tank. From this plant two samples were obtained; (1) raw sewage before treatment by the septic tank, and, (2) effluent from the septic tank. Filtrates of these two sewage samples were added to freshly inoculated beef infusion broth cultures of a series of organisms isolated from the raw sewage and consisting of eight cultures of organisms producing acid from lactose together with five which could not split this sugar. Results are shown in table 3.

These samples were taken at a time when there was no unusual load upon the plant. A decided degree of activity is shown,

particularly upon those organisms inactive upon lactose litmus plates.

Experiment IV. Sewage from the City of Santa Rosa, California, passes through a septic tank, then through a deep baffle chamber known as the "Dark Tank." In turn it is then exposed to oxidation for some days in a series of shallow ponds. Finally it is chlorinated, at the rate of one part chlorine to approximately 800,000 parts of sewage before passing into a nearby creek. Samples were taken as follows: (1) Raw sewage before entering the septic tank. (2) Effluent from septic tank. (3) Effluent from "Dark Tank." (4) Effluent from one of the settling and

TABLE 3

Bacteriophage action of Sonoma Home sewage upon organisms isolated from that sewage following twenty-four hours incubation at 30°C.

CULTURES	RAW SEWAGE FILTRATE	SEPTIC SEWAGE FILTRATE	CULTURES	RAW SEWAGE FILTRATE	SEPTIC SEWAGE FILTRATE
A1	−	−	B1	+++	+++
A2	++	−	B2	++++	++++
A3	+++	++	B3	+++	+++
A4	−	−	B4	++++	+++
A5	++	−	B5	++++	++++
A6	+	−			
A7	+	−			
A8	−	+			

− = no inhibition; + = very slight lysis as indicated by zones of clarity or slightly decreased turbidity; ++ = more evident inhibition; +++ = moderate lysis; ++++ = complete lysis.

oxidizing ponds. (5) Final effluent from the system thirty minutes after chlorination at rate of 1:800,000. In preparation for these tests, a series of seven acid producers upon a lactose medium and five inactive against this sugar were isolated from the raw sewage. The sewage samples listed above were filtered. This experiment was conducted by bringing 1 cc. quantities of the several filtrates into contact with freshly inoculated cultures of these organisms in beef infusion broth. Results are indicated in table 4.

The results set forth in table 4 are of marked interest because of the intense lytic activity manifested by certain of the sewage

filtrates against a number of the organisms derived originally from the system. This is shown with the lactose splitting forms A4 and A5 especially and again with the strains inactive towards lactose noted by B before the numeral. Next, the potency of these several filtrates is not the same throughout the system. Finally, it is of intense interest to observe that the heavy dosage of chlorine administered in most instances did not destroy bacteriophage within the sewage. This reveals marked resistance on the part of many strains of bacteriophage to the effects of

TABLE 4

Lytic activity of Santa Rosa, California, sewage against organisms isolated from the raw sewage itself, following twenty-four hours incubation at 30°C.

CULTURE	RAW SEWAGE FILTRATE	SEPTIC SEWAGE FILTRATE	EFFLUENT FROM "DARK TANK"	EFFLUENT FROM OXIDIZING POND	EFFLUENT AFTER CHLORINATION
A1	−	+	−	+++	++
A2	+	+	+	+	+
A3	+	+	+	+	+
A4	+	+	+	+++	+++
A5	++	+++	+++	+++	+++
A6	+	+	++	+	−
A7	+	+	−	++	+
B1	++	+++	++++	+++	++++
B2	+++	+++	++++	++	++++
B3	+++	+++	++++	++	++++
B4	++	+++	++++	+	++++
B5	++	++++	++++	+++	++++

− = no inhibition; + = very slight lysis as indicated by zones of clarity or slightly decreased turbidity; ++ = more evident inhibition; +++ = moderate lysis; ++++ = complete lysis.

chlorine. In one instance only (A6) was lytic action destroyed following chlorine administration.

Experiment V. It was desired to examine sewage from the Santa Rosa, California, plant following the rainy season in a manner similar to that followed with material from Sonoma outlined in experiments I and II. Thus, shortly after the stormy period of the winter's rains, two additional samples were obtained from this source. These consisted of: (1) raw sewage before entering the septic tank; and (2) effluent after treatment by the septic tank. Fourteen acid producing organisms on lactose lit-

mus agar and thirteen forms inert towards this carbohydrate were isolated from the crude sewage sample, and cultures of each of these were treated with the filtrate of the sewage from the septic tank as outlined previously. Results are indicated in table 5.

It thus appears that in sewage there are a considerable number of strains of bacteriophage which are potentially very active against the contained intestinal organisms. This inhibition here

TABLE 5

Lysis induced by Santa Rosa, California, sewage following flushing by rains. Incubation twenty-four hours at 30°C.

CULTURE	SEPTIC SEWAGE FILTRATE	CULTURE	SEPTIC SEWAGE FILTRATE
A1	−	B1	−
A2	+	B2	−
A3	+++	B3	−
A4	−	B4	−
A5	+++	B5	+++
A6	+	B6	++
A7	−	B7	−
A8	+++	B8	+
A9	−	B9	+
A10	−	B10	+
A11	+++	B11	−
A12	+++	B12	−
A13	+++	B13	−
A14	−		

− = no inhibition; + = very slight lysis as indicated by zones of clarity or slightly decreased turbidity; ++ = more evident inhibition; +++ = moderate lysis; ++++ = complete lysis.

demonstrated is not due to the presence of some chemical factor for upon numerous instances we have "stepped it up" by procedures which are well understood by those who have had occasion to work with bacteriophage. Thus, the initial degree of activity is greatly increased by passage. We have proved that these strains of the lytic agent show definite inhibitive effects upon some of the organisms of sewage. It appears likely that this power to lyse is effective in some degree also in reducing the bacterial content of sewage under natural conditions, although there is no reason to suppose that the activity of bacteriophage is

as efficient here as when the technique is carried out with rapidly growing cultures. In sewage the content of young organisms is less and temperature relationships are not fulfilled to the optimum. It does appear reasonable to assume however, that the contained strains of bacteriophage in sewage constitute an agency having a certain degree of effectiveness in reducing the numbers of organisms present.

CONCLUSIONS

1. The strains of bacteriophage in sewage are effective against many bacterial organisms of the sewage itself. Those organisms inert towards lactose are relatively somewhat more susceptible to sewage phages than those which are able to split this carbohydrate.

2. The content of bacteriophage varies with seasonal conditions: it is diminished following the flushing induced by heavy rains. Thus, it is similar in this respect to the bacterial content.

3. Many, but not all, strains of bacteriophage are resistant to chlorine as applied in the chlorination treatment of sewage.

4. Although conditions in sewage are not optimum for multiplication and adaptation of bacteriophage to the various organisms present, it appears likely that the bacteriophage strains present are effective within certain limitations not only in inhibiting bacterial growth but even in bringing about some degree of diminution of the intestinal bacterial flora therein.

Thanks are extended both to Mr. P. F. Bovard of the California Filter Company of San Francisco, California, and to Mr. C. G. Gillespie of the California State Board of Health. Through their courtesy, it was possible for us to obtain certain of the samples herein reported.

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FACTORS WHICH INFLUENCE TESTS OF BACTERIAL SURVIVAL

I. THE EFFECT OF VARYING PERIODS OF INCUBATION OF THE SURVIVAL TEST CULTURE

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In the determination of the effect of heat or chemicals on a given suspension of bacteria it is usually assumed that the concentration of the disinfectant, if chemicals are used, the temperature and the time of exposure are the only factors involved. In the usual procedures the culture is heated or placed in contact with a disinfectant and after varying periods, portions of it are inoculated in agar or broth. These survival test cultures are incubated, usually at 37°C., for an arbitrary length of time, in most instances two days, when the results are read. It is not usually recognized that if another medium is used for the survival test or if a different incubation time is chosen the results may be significantly different. Other factors, such as the temperature of incubation after heating are probably also of importance.

In this paper we shall deal with the effect of the incubation time on the results of the survival test and reserve a consideration of the nature of the media used for a following paper.

Some of the results reported in the literature are of interest. Eijkman (1908), in obtaining data on the death rate of a strain of *B. coli*, observed that counts made after six days of incubation were distinctly higher than those made after two. The organisms were apparently "killed" in the first case in three minutes and in the second in five minutes. In another experiment, where counts were made after three and fifteen days, apparent sterility was obtained in the first count in the cultures made after five minutes of heating, while in terms of the second count, more than thirty-

five minutes of heating were necessary. These figures show that the longer the exposure to heat, the greater was the percentage

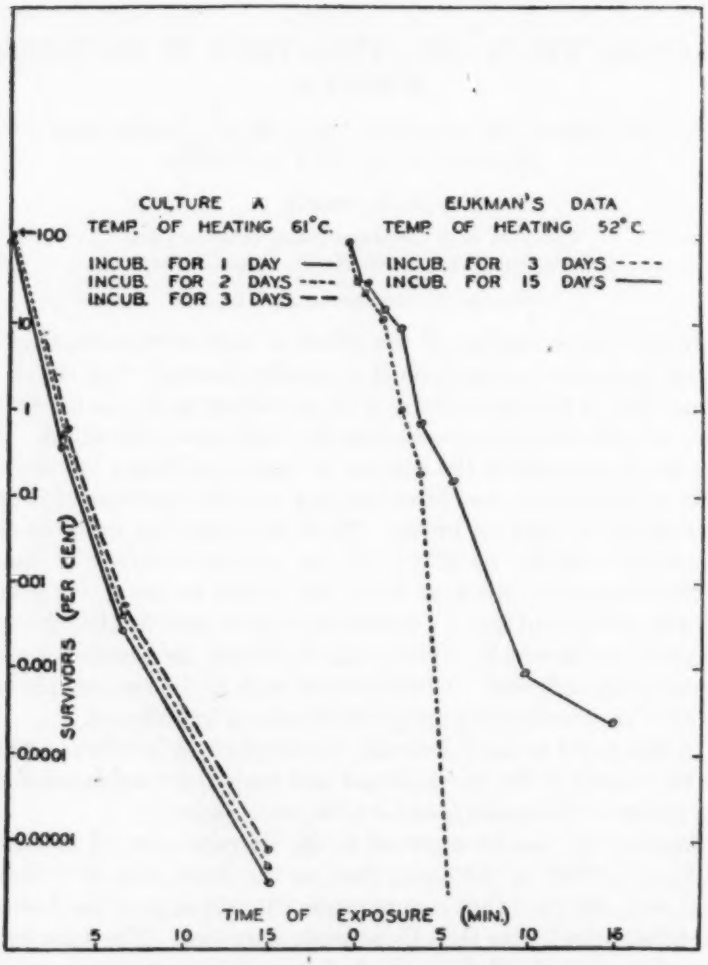

FIG. 1. THERMAL DEATH CURVES OF B. COLI AFTER VARYING PERIODS OF INCUBATION OF THE SURVIVAL TEST CULTURES

of organisms which developed after a lag. The unheated cultures in each instance gave their maximum counts after the shorter period of incubation. The results are plotted in figure 1.

Experiments on the tubercle bacillus were performed by Oerskov (1925) with interesting results. A suspension was heated to 63°C. and portions transferred to Petroff's medium at five-minute intervals. The tubes were examined after one month and three months of incubation. Five tubes were made for each time interval. The results after one month of incubation of the survival test culture indicated some survival among the organisms heated for thirty minutes but none in the culture exposed for thirty-five minutes. After three months the forty-minute cultures were all positive. The forty-five-minute tubes were still sterile at this time. The author does not indicate how long an unheated culture required before its growth could be observed. The results, however, clearly indicate that thirty minutes of heating at 63°C. was sufficient to "kill" the culture if the thirty day results are considered alone, whereas the three-month record shows that the organisms were viable for an additional ten minutes. Delepine (1916) has reported experiments with milk containing tubercle bacilli which was heated and injected into guinea pigs. The unheated culture produced symptoms within two weeks. Milk which had been heated to 78° or 92°C. and cooled, produced no local lesions on injection but at the end of the eleventh week tuberculous lesions were found in various organs. Tubercle bacilli were also found, but inasmuch as these were not demonstrated to be living, it cannot be said definitely that the experiments demonstrated a delay in infection caused by the introduction of temporarily injured bacteria. The observations by Lange (1923) on mice injected with various types of treated organisms are more positive and indicate that such latent infections are possible.

The lag before growth which is observed after spore formers have been heated, has been the subject of some study. The original observation in this connection was made by Koch in 1881. Most of the work has been done on the spores of the botulinus organisms (Tanner and McCrea (1923), Dickson, Burke, and Ward (1919), Burke (1918 and 1923), Esty and Meyer, (1922), Weiss (1921), and Starin (1926). In general it has been found that high temperatures injure some of the spores to such an

extent that lag periods of as much as several months may be observed. Miss Burke found, for example, that with a series of unheated cultures the maximum germination time was 144 days, whereas for the same cultures heated, the maximum germination time was 214 days. She states that cultures made from heated spores "remain apparently sterile for days. No growth is visible even with the use of the dissecting microscope. Then suddenly, within a twenty-four- to forty-eight-hour period, normal growth and gas production take place." She also observed that the lag period varies directly with the length of exposure.

It is difficult to decide whether the lag period is due to a lapse of time before the individual which has been heated begins to to grow or whether it and its immediate descendants are permanently changed and grow continuously but slowly. Allen (1923) has reported some experiments which tend to show that in some cases the rate of growth of heated organisms is slower than that of the same organisms unheated. He points out, however, that inasmuch as his heated organisms grew in the presence of large numbers of dead organisms, and the unheated in a fresh medium, the results may not be comparable. If the lag period with which we are dealing is similar to that of organisms which have been transplanted after the logarithmic phase of growth, then it definitely involves the first organism or its immediate descendants. Our own observations[1] lead us to the view that the injury caused by heat is limited to the organism heated or to the first few generations derived from it.

The nature of the medium used for the survival test has some effect on the lag period. The observations of Lange (1923) on staphylococci disinfected with 0.4 per cent tricresol afford an interesting example of this. With horse meat broth, with or without glucose as the survival test medium, exposure to the

[1] We have observed that once a colony has started to develop, it grows as rapidly as one from an unheated organism. The following observation is also suggestive. A typhoid suspension was heated for thirty minutes at 52°C. On plating, one colony developed after a lag of twenty-four hours. This was demonstrated to be typhoid by agglutination tests. On inoculation in broth the same maximum number of organisms developed as in the original unheated culture and the death curves for the two were almost identical.

disinfectant for more than ten minutes caused a lag of more than twenty-four hours, while, with a serum broth, such a lag was absent up to fifty minutes of exposure. Miss Burke, working with botulinus spores, noted that the maximum germination times were dependent to some extent on the medium. For heated cultures incubated in sealed tubes of broth the time was 214 days. In agar the time was 201 days. If the broth tubes were layered with oil the maximum germination time was 426 days.

In order to account for the lag phenomenon Miss Eckelmann (1917–1918) has advanced a rather ingenious theory. She supposes that in any suspension there are a small number of organisms with thick surrounding membranes. Handicapped by the membrane, such organisms grow very slowly. She assumes that the membrane also renders them more resistant, so that, after the application of heat to a suspension, the relative numbers of such organisms among the survivors increase, the proportion rising with prolonged heating. The theory serves to explain many of the facts but there are two factors which militate against its acceptance. In the first place, it is not clear why a thick surrounding membrane should render organisms less sensitive to heat. It can be calculated[2] by Fourier's law of heat conduction that the time required for a ninety-nine per cent temperature equalization between the outside and inside of a cell through a membrane 1 micron thick would be less than 0.1 second, assuming the membrane to be such an extremely poor conductor as pine wood measured cross-grain. In the ordinary procedures a period of time of this magnitude would be of no significance. The second objection concerns the nature of the curves obtained. According to the theory we can assume that a given suspension consists of two types of organisms, a non-resistant and a resistant form. With not too vigorous procedures, the following results should be obtained. With a short incubation period of the cultures we should expect the results when plotted to give the usual logarithmic curve. Upon prolonged incubation, another curve, also logarithmic, should appear, beginning more or less sharply toward the end of the first curve and declining less

[2] I am indebted to Professor Earle B. Phelps for this computation.

steeply than the first. Examination of a series of death curves such as those of Chick, in which in all probability a sufficiently long incubation period was used, show very few curves of this description. Our own data do not indicate a mixture of organisms such as the theory implies.

Our observations on the lag period were made during the course of a series of experiments designed to obtain data on the thermal death of several kinds of organisms. They pointed to several conclusions of interest and likewise suggested a theory as to the mechanism involved in the lag.

EXPERIMENTS WITH B. COLI

Two types of experiments were performed with *B. coli* cultures. In the first, a suspension was held at 61°C. for varying periods of time, then cooled and plated. The results of two experiments with two different cultures are given in table 1 and those of the first are shown in the graph (fig. 1). The numbers in parenthesis are the actual plate counts.

On the twenty-four-hour plates there were in many cases small colonies which were rather pale in appearance. It is highly probable that in many cases small particles of precipitate in the media or imperfections in the glass of the petri dish were confused with these.

It will be noted that the increases are not very great in any case; but it is clear that, in general, the longer the heating the greater the percentage increase in the number of colonies from day to day. In most cases, also, the increase of the third count over the second is less than that of the second over the first. It will be noted also that the unheated cultures show an increase in count on prolonged incubation. For comparison the curves from Eijkman's figures are also plotted.

In another series of experiments a suspension of *B. coli* was heated in a special apparatus, which will be described in another place, for 0.2 second and rapidly cooled. The results are given in the table 2.[3] It appears from these data that the higher the temperature the greater is the percentage of delayed colonies.

[3] I am indebted to Miss Marion Adkerson for help in obtaining these data.

Incubation for an additional twenty-four hours after the second day increased the counts very slightly. This agrees with our conclusions in the first experiments.

TABLE 1

	MINUTES OF HEATING	COLONIES ON PLATES AND SURVIVORS PER CUBIC CENTIMETER OF ORIGINAL SUSPENSION. INCUBATION OF SURVIVAL TEST CULTURES FOR:					
		24 hours		48 hours		72 hours	
Culture A	0	(140)	700,000,000	(140)	700,000,000	(148)	740,000,000
	3½	(356)	3,600,000	(388)	3,880,000	(402)	4,020,000
	6½	(21)	21,000	(35)	35,000	(38)	38,000
	15	(26)	26	(37)	37	(59)	59
Culture B	0	(22)	110,000,000	(27)	135,000,000	(33)	165,000,000
	3	(27)	270,000	(34)	340,000	(36)	360,000
	6	(15)	15,000	(29)	29,000	(32)	32,000
	10½	(26)	260	(41)	410	(42)	420
	15	(4)	40	(4)	40	(8)	80
	20	(9)	9	(10)	10	(13)	13
	26	(1)	1	(1)	1	(5)	5

TABLE 2

TEMPERATURE	COLONIES ON PLATES AND SURVIVORS PER CUBIC CENTIMETER OF ORIGINAL SUSPENSION. SURVIVAL TEST CULTURES INCUBATED FOR:					
	24 hours		48 hours		72 hours	
°C.						
Room	(42)	42,000,000	(43)	43,000,000	(44)	44,000,000
78.2	(74)	74,000	(78)	78,000	(87)	87,000
78.4	(65)	65,000	(81)	81,000	(81)	81,000
79.3	(197)	19,700	(270)	27,000	(286)	28,600
80.2	(34)	3,400	(66)	6,600	(73)	7,300
80.8	(81)	8,100	(128)	12,800	(142)	14,200
82.3	(25)	250	(88)	880	(92)	920
82.5	(142)	142	(452)	452	(516)	516
83.5	(0)	0	(10)	10	(18)	18

EXPERIMENTS WITH A HEMOLYTIC STREPTOCOCCUS

The results obtained when a hemolytic streptococcus was used to study lag were more clear-cut, since the zones of hemolysis on blood plates made the colonies more easily recognizable.

Another advantage was that the organism, although growing in clumps in broth, suspended very easily. The suspensions were of surprising uniformity and duplicate counts checked very satisfactorily. Our first culture[4] was obtained from the pleural fluid of a rabbit, an animal for which the organism was particularly virulent. The data for the second and fifth broth transfers are given in table 3 and figure 2. The suspensions of the organ-

TABLE 3

	MINUTES OF HEATING	COLONIES ON PLATES AND SURVIVORS PER CUBIC CENTIMETER OF ORIGINAL SUSPENSION. INCUBATION OF THE SURVIVAL TEST CULTURES FOR:					
		24 hours		48 hours		K* 24 hours	K 48 hours
Transfer II	0	(22)	22,000,000	(28)	28,000,000		
	2	(32)	3,200,000	(34)	3,400,000	0.963	1.06
	3	(32)	320,000	(124)	1,240,000	2.30	0.889
	5	(34)	34,000	(45)	45,000	1.07	1.55
	9	(1)	1	(33)	33	2.63	1.82
Transfer V	0	(18)	1,800,000	(18)	1,800,000		
	3	(21)	210,000	(50)	500,000	0.718	0.428
	6	(43)	43,000	(111)	111,000	0.530	0.503
	10	(6)	600	(155)	15,500	1.08	0.496
	15	(24)	240	(336)	3,360	0.183	0.305

* Calculated as a K for a monomolecular reaction using the equation

$$K = \frac{2.3}{t_2 - t_1} \log \frac{C_1}{C_2},$$

where C_1 and C_2 are the number of organisms surviving at times t_1 and t_2, respectively.

isms were made in saline and the heating was carried out at 50°. The survival test culture was made in blood agar and the plates were incubated at 37°C. The appearance of the plates after various periods of incubation may be summarized as follows.

A. Plates made from unheated suspensions

Incubation for twenty-four hours. Well developed colonies of uniform size and sharply defined zones of hemolysis have appeared. *Incubation for forty-eight hours.* The colonies grow

[4] This was kindly given to us by Dr. A. R. Clark.

somewhat larger. Few, if any, new colonies appear. *Incubation for seventy-two hours.* The zones of hemolysis increase consider-

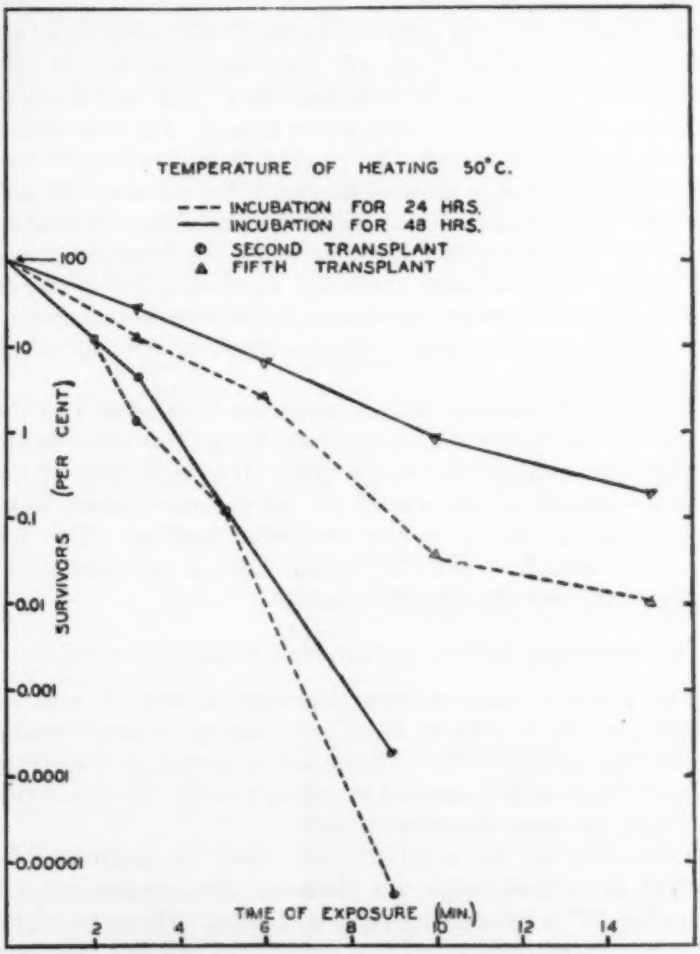

FIG. 2. THERMAL DEATH CURVES OF A STREPTOCOCCUS CULTURE AFTER ONE AND TWO DAYS' INCUBATION OF THE SURVIVAL TEST CULTURE

ably producing overlapping in the more crowded plates. In the few plates counted at this time no new colonies were found.

B. Plates made from heated suspensions

Incubation for twenty-four hours. A few well developed colonies, resembling in size and appearance those on the control plates, are present. In some cases a few ill-defined colonies with very faint zones of hemolysis are seen, but these do not occur frequently. *Incubation for forty-eight hours.* The well developed colonies of the day before have grown larger. The faint colonies develop and are surrounded by sharply defined hemolytic zones almost equal in size to those of the rest of the colonies. In addition new colonies have sprung up on the plates and are clear cut. They are usually somewhat smaller than the twenty-four-hour colonies of the unheated controls. *Incubation for seventy-two hours.* There is usually no change, aside from the increase in size of the hemolytic zones. Occasionally, a new colony may be found on a plate.

From this description and an inspection of the data it will be noted that the longer the heating time the greater the difference between the one and two day counts. It is of interest to note that the counts of the second day give a curve more nearly approximating that of a monomolecular reaction. This may be readily seen from the "K" values given in the table and is particularly evident in the fifth transfer.

EXPERIMENTS WITH A SAPROPHYTIC TUBERCLE BACILLUS

The organism used in these experiments was an acid fast bacillus, which is said to have been derived from a virulent culture isolated by Koch. After many thousands of transplants it has become so well adapted to ordinary media that incubation overnight produces abundant growth.

Suspensions of this organism were made by grinding a few loopfuls in milk or broth and filtering. The suspensions were heated to 60°C. for varying periods of time. The survival test cultures were made by pouring or spreading 1 or 0.5 cc. on poured agar plates. The results of a typical experiment are given in table 4. It will be noted that the colonies developed from the unheated suspension in twenty-four hours. In forty-eight hours they had

increased to such size that they became confluent and covered the plate. One minute of heating resulted in a few colonies which could not be counted because they were confluent. In another twenty-four hours many more had appeared and the original colonies had grown very large. In twenty-four hours no growth was observed on the plates made after five minutes of heating but

TABLE 4

MINUTES OF HEATING	COLONIES DEVELOPING ON SURVIVAL TEST CULTURE PLATES AFTER THE FOLLOWING TIMES OF INCUBATION			
	24 hours	48 hours	72 hours	96 hours
0	About 300 colonies	Plate covered	Plate covered	Plate covered
1	A few colonies (confluent)	Plate covered	Plate covered	Plate covered
5	No growth	A few colonies	Plate covered	Plate covered
10	No growth	No growth	Many colonies	Plate covered
15	No growth	No growth	4 colonies	8 colonies
20	No growth	No growth	No growth	1 colony
25	No growth	No growth	No growth	No growth
30	No growth	No growth	No growth	No growth

TABLE 5

	MINUTES OF HEATING	COLONIES ON PLATES AND SURVIVORS PER CUBIC CENTIMETER OF ORIGINAL SUSPENSION. INCUBATION OF THE SURVIVAL TEST CULTURES FOR:		
		72 hours	96 hours	144 hours
Suspension A	0	(70) 700,000		(77) 770,000
	2	(8) 8,000		(13) 13,000
	6	(13) 1,300		(37) 3,700
Suspension B	0	(16) 16,000	(18) 18,000	
	2	(0) 0	(14) 28	

a few colonies appeared after forty-eight hours. These increased in number and size on additional incubation. Ten minutes of heating resulted in a lag greater than forty-eight hours, while a twenty minutes exposure prevented growth for more than seventy-two hours. Twenty-five minutes exposure prevented any growth for at least ninety-six hours.

In two other experiments in which the colonies were discrete and therefore could be counted, the results were as shown in table 5.

The conclusions to be drawn from these experiments are similar to those which were suggested by the work on *B. coli* and the hemolytic streptococcus. In this case, however, the lag is considerably longer and the recovery of the organisms more gradual.

DISCUSSION

From the experiments reported in this paper it will be seen that heat may fail to affect an organism or may so change it that it fails to grow on a given medium or may produce visible growth only after the lapse of some time. The longer the exposure to a high temperature the greater the number of organisms partially injured. These results were found to be common to three widely different types of organisms so that they may be assumed to have a general application. In addition, the data on *B. coli* showed that, the lower the temperature, the smaller the difference between the number of organisms apparently killed and the number showing lag.

The death of a bacterium would appear to be, then, a gradual or catenary process. At the present time we are unable to follow the course of destruction; we are merely able to say that after a given treatment a certain percentage of organisms are dead *in terms of a given survival test medium* and in terms of a *given incubation time*. We are unable to say whether a given medium can cause survival of, let us say, a half-dead organism or of an organism one-third dead or what length of time is necessary to cause the revival of such organisms. The death of a micro-organism, in so far as we are able to determine, is an arbitrary point definable, in part at least, by the conditions under which survival is tested. There must be, of course, a limit in the destruction beyond which no medium and no length of time of incubation can cause survival. This limiting condition corresponds to our ordinary conception of death in higher animals.

If the bacterial cell were composed of a definite number of

identical molecules it would not be difficult to account for the facts, particularly those observed in connection with the lag period. Such a picture can hardly be a true one, however. As an alternative we may make the assumption that there exist in the bactrial cell, among the various types of molecules, a number (not necessarily all of one kind) which may be looked upon as a measure of the organism's life and that as these are destroyed or removed the organism is brought progressively nearer death. In order to obtain a picture in concrete terms, we might think of the molecules of the bacterial enzymes as being the units in question. There is some evidence for such being the case, but a consideration of the theory must be left for another place.

SUMMARY

Suspensions of *B. coli*, a streptococcus and a saprophytic tubercle bacillus were heated for various periods of time at fixed temperatures and plated on agar. The colonies developing were counted after one, two, three or more days. There was an increase in the counts from day to day, the greatest relative increases occurring in the plates made from cultures heated the longest. The greatest increases in counts occurred with *B. coli* and the streptococcus between twenty-four and forty-eight hours. The results with the saprophytic tubercle bacillus showed a more gradual recovery of the organism, the counts increasing up to four days. In the case of *B. coli* heated to different temperatures, it was found that, the higher the temperature, the greater was the relative number of delayed colonies. Unheated cultures usually developed their maximum numbers in twenty-four hours.

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FACTORS WHICH INFLUENCE TESTS OF BACTERIAL SURVIVAL

II. THE EFFECT OF THE pH OF THE SURVIVAL TEST CULTURE ON RESULTS OBTAINED WITH A SAPROPHYTIC TUBERCLE BACILLUS

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In the preceding article it was pointed out that in addition to time and temperature, other factors are of importance in the determination of the thermal death of an organism. It was shown there that one of these factors is the length of time for which the survival test cultures are incubated. The experimental results indicated that heat causes a delay in the germination of some of the organisms exposed. The longer the application of heat, and the higher the temperature, the greater is the relative number of organisms starting to develop after a period of lag. In the present paper another factor, namely, the composition or nature of the medium on which the survival test culture is made, will be considered.

After a culture has been heated or exposed to chemicals there are several ways in which survival or non-survival may be tested. The culture may be fed to or injected into an animal, or it may be put into a variety of artificial culture media. An examination of the literature shows that little if any correlation exists between the results of the various procedures.

In comparing results of animal inoculation with growth on artificial media one would expect that the heat-injured organisms would fall an easy prey to the leucocytes and therefore that the use of artificial media would be a more delicate test for survival. Such has been found to be the case by Lange, (1921) Morgenroth

(1923) and others. There are, however, several workers whose observations have led them to quite different conclusions. Mueller (1923) found that anthrax spores may, on treatment, give negative cultures and yet be able to kill animals. He calls attention to a similar observation made by Geppert in 1889. The results obtained by Hahn (1922) lead to the same general conclusions. Various species and disinfectants were used. The experiments with the anthrax and streptococcus organisms with mercuric chloride may be cited as examples. With a 1:100 dilution of the sublimate, streptococci were killed, in terms of artificial media, in 15 minutes, although still infective for animals after approximately one hour's contact with the disinfectant. A suspension of anthrax, when treated with 1:1000 bichloride, survived one day, according to the results on artificial media, although three days of exposure were necessary to prevent fatal infections in animals. Neufeld (1923), in preparing plague vaccine, came to the conclusion that a treated suspension of *B. pestis* may fail to give growth on media but yet kill guinea pigs. He points out however that the media, although satisfactory for the unheated organisms, may not have been favorable for those injured by heat. Similar conclusions were reached by Bleyer (1923) with the streptococcus. It must be remembered, that aside from the fact that a proper agar medium may not have been chosen, the lag period induced by heating or chemical treatment may not have been taken into account in the experiments cited. In some instances, also, it appears that lesions may have been produced by dead organisms.

North and Park (1927) have recently called attention to the added margin of safety which is given by the use of subcutaneous inoculation of guinea pigs as a means of testing for the survival of the tubercle bacillus in experimental pasteurization procedures. They refer to the work of Bang (1902) who found that suspensions of tubercle bacilli were killed, in terms of feeding experiments, by exposure to 140°F. for two minutes. North and Park, (1927) using subcutaneous inoculation, found that ten minutes were required at that temperature to prevent infection. We may mention here for comparison the results of Oerskov (1925)

who, working with artificial media, found that as long as forty-five minutes were required at 145.4°F. These comparisons are of course valid only if comparable numbers of organisms were used by each worker.

Comparative data on different kinds of media have been collected by Süpfle (1915) and Lange (1921). Both found that the apparent survival was increased to some extent by the addition of sugar to the survival test medium. The best results, however, were obtained when serum was present. Using a suspension of *Staphylococcus aureus* and the tricresol, Lange found that with horse meat broth as the survival test medium there was a survival up to forty minutes of contact; with the same medium containing glucose there was survival for fifty minutes, while in a serum broth, growth occurred after seventy minutes, of previous contact between disinfectant and organism. Süpfle also used various organisms and disinfectants. In general the presence of 3 per cent of glucose in broth approximately doubled the apparent survival time. In the case of *B. coli* treated with 1 per cent phenol there was survival for thirty minutes when the survival test culture was made in broth and for ninety minutes when the final test culture was made in glucose horse serum broth. It would appear from these results that when an enriched medium is used for the survival test there is an apparent increase in the length of time required for the disinfection process, or in other words, such a medium permits partially killed organisms to survive.

In carrying out some experiments designed to determine the thermal death curves of a saprophytic tubercle bacillus some chance results were obtained which throw some light on the influence of the survival test media on the survival or death of an organism.

The organism[1] used in these experiments was the same as that used in the experiments reported in the previous paper. On ordinary nutrient agar abundant growth is obtained in twenty-four hours. Suspensions were prepared by grinding portions

[1] The organism is probably the same as "Mycobacterium tuberculosis number 607" of the American Type Culture collection.

of a forty-eight-hour agar growth in buffer solution of pH 6.8 and filtering the suspension.

In general, the results obtained when suspensions were heated were similar to the results with other organisms. Heating caused a lag in the appearance of colonies on agar plates. The longer the heating the greater was the lag. Typical results are given in the previous article.

In one experiment a curious result was obtained. A suspension of the organism had been heated and portions of it, in duplicate, spread on prepared agar plates. In one set, one plate remained sterile while, on the other, many colonies of the tubercle bacillus appeared. On the plate containing the colonies there was a thin transparent growth of a contaminating organism.

The experiment was repeated with some plates inoculated with the contaminant. It was found that wherever it was added, the number of colonies of the tubercle bacillus was greatly increased. The results of a typical experiment with the suspension heated to 60° for two and one-half minutes were as follows, the results being read after two days of incubation.

Plate without contaminant: 49 colonies, all small, the largest 1 mm. in diameter.

Plate with contaminant: 157 colonies, of varying sizes, many 3 mm. in diameter.

The contaminating organism fulfilled the characteristics of *B. zopfius* and a culture of this organism kindly sent to us by Dr. Leo F. Rettger, although differing in some characteristics from ours, was found to have the same adjuvant effect.

Several possibilities suggested themselves as to the action of the *B. zopfius*. It appeared probable that through its presence the proper conditions of oxygen tension for the growth of the injured organisms were brought about or that it furnished certain necessary food materials which, although not necessary for the unheated tubercle bacilli, were required for those whose functions had been disturbed by exposure to heat. While experiments were in progress to determine whether these factors were

operative, a second chance occurrence pointed to the solution of the problem.

In repeating the experiment on one occasion, it was found that the plates without *B. zopfius* produced colonies almost as large and equal in number to those on the plates containing the organism. An examination of the medium showed that it was very alkaline, pH 7.8 to 8.0. The media in the previous experiments had been about pH 6.6. The experiments were repeated again with all of these factors taken into consideration and the results

TABLE 1

	COUNT	
	After 5 days	After 8 days
Agar I, pH 6.6		
Culture unheated 1:100,000	40	44
Culture unheated 1:100,000 + *B. zopfius*	59	59
Culture heated to 60° for 10 minutes	0	0
Culture heated to 60° for 10 minutes with *B. zopfius* inoculated on plate	0	20
Agar II, pH 7.6		
Culture unheated	27	30
Same with *B. zopfius* on plate	26	46
Culture heated as above	0	4
Culture heated as above, *B. zopfius* on plate	0	5

shown in table 1 obtained. By placing drops of indicator on the agar plates the following results were obtained:

Agar I

pH of agar = 6.6
pH of agar in vicinity of colonies of T.B. = 7.8
pH of agar in vicinity of *B. zopfius* areas = 7.8–8.0.

Agar II

pH of agar = 7.6
pH of agar in vicinity of colonies of T.B. = 7.8
pH of agar in vicinity of *B. zopfius* areas = 7.8–8.0

The facts which these experiments brought out were as follows. The acid fast organism can grow on either acid or alkaline media.

In growing it produces sufficient alkali to bring the medium to a pH of about 7.8. When a suspension of the organism is heated, its ability to produce alkali is destroyed or injured. It, then, may fail to grow on an acid medium. An alkaline medium, whether initially alkaline or made so by some other means such as by the growth of *B. zopfius*, enables some of the organisms to grow. It is interesting to note that the lag period due to heating is not reduced by supplying an alkaline medium for growth. It is, of course, possible that if we had continued the incubation of the acid plates for some months additional colonies would have appeared. In such a sense the period of lag was reduced by the more alkaline medium.

DISCUSSION

From a consideration of our own data and those of others it will be seen that an organism which has been exposed to heat or chemicals may be so injured that it may find the media on which it originally grew unsatisfactory for further growth. On another media, growth may be excellent. Animal inoculation may be a more, or a less, sensitive test depending on the nature of the organism. It is difficult at the present time to picture just what changes take place in the organism or what differences exist in media to account for the phenomenon. Our own experiments show that the pH of the survival test medium is a factor, at the same time indicating that one change produced in the saprophytic tubercle bacillus by exposure to heat is the destruction or diminution of the ability to produce alkali. Since the production of alkali is without doubt an enzymatic process, the conclusion is almost inescapable that here, at least, the effect of heat is to destroy or injure certain enzymes of the cell.

SUMMARY

A saprophytic tubercle bacillus was found to grow well on agar of pH 6.6. When a suspension of the organism is heated the number of survivors able to grow on the same medium is decreased. After heating, growth occurs after a period of lag. When cultivated on an alkaline medium of pH 7.6 to 7.8 more

organisms survive, although the lag period is apparently not reduced. This pH may be obtained either through the growth of *B. zopfius* or by the addition of alkali. The unheated organism, in growing, produces an alkaline reaction in an originally acid medium.

The explanation of the phenomenon probably lies in the fact that heat destroys certain enzymes of the organism which produce alkali.

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SONNE DYSENTERY

A REPORT OF 32 CASES OF DYSENTERY CAUSED BY EBERTHELLA PARADYSENTERIAE SONNE

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During the past six months cases of dysentery and mild diarrheal disease have been encountered, caused by a late-lactose fermenting bacillus, which in its early cultural and fermentative reactions so closely resembled *E. dysenteriae* Flexner that it was at first regarded as a member of that group of organisms. Attention was particularly directed to a study of the organism by the regularity with which it fermented rhamnose, a characteristic possessed only by the rarer strains of Flexner bacilli, and by its being inagglutinable in stock Flexner and polyvalent dysentery anti-serums. After twenty-four hours incubation, the organism in question fermented glucose, mannitol, maltose, and rhamnose, and failed to ferment lactose, xylose, sucrose and dulcitol. After two to six days incubation, acid was formed in lactose, the other sugars remaining as mentioned. In no instance was gas formed in any sugar. Because of these characteristics, and because of the large numbers in which the organisms were present in the stools of the cases studied, the bacillus was subjected to a more careful study, the results of which will be given later. It was identified as a strain of *E. dysenteriae* Sonne, which heretofor has been little seen in this country.

The association of late-lactose fermenting dysentery-like organisms with diarrheal disease was first noted by Duval and Schorer (1904), who found these organisms while studying summer diarrhea in infants. The organism described by them produced a primary acidity in litmus milk which was replaced by alkalinity

after forty-eight hours, with a second wave of acidity following after five or six days, this last wave being permanent and more intense than the primary. They described two strains of organisms, one fermenting glucose, mannitol and lactose, but not dextrin, which they called Bacillus "A"; and another, which they called Bacillus "B," which fermented dextrin in addition to the other sugars. Duval (1904) isolated a similar strain of organism in large numbers from the contents and scrapings of the gut in a fatal case of dysentery in an adult. In his case, the lower portion of the colon was the site of involvement, with a pseudo-membrane present. The mucosa showed many small necrotic areas. The microorganism isolated acted the same in milk as did those studied by Duval and Schorer. Glucose, mannitol, maltose, lactose, dextrin, and galactose were acidified without gas production, while sucrose and inulin were not fermented. The bacillus was agglutinated by the patient's serum in a dilution of 1:400, by Flexner-Harris dysentery anti-serum 1:200, by typhoid anti-serum 1:80, and not at all by Shiga dysentery anti-serum. Duval believed the organism closely related to *E. typhosus*. Torrey (1905) reported the finding of similar microorganisms in the stools of infants with diarrhea. Although several of the infants in question died, he would not admit an etiological relationship, because in the few instances in which the test could be done, the patient's serum either failed to agglutinate the bacillus or did so in low dilution only.

Sonne, in 1915, found that the main causative agent in dysentery in Copenhagen was a late-lactose fermenting bacillus which he showed to be serologically specific and different from any other type of dysentery bacillus. Following Sonne's description of the bacillus, d'Herelle (1916) in France also found this atypical organism associated with cases of dysentery.

Andrewes (1918), in an attempt to differentiate the true dysentery bacilli from the allied species, suggested the name *B. dispar* for lactose fermenting organisms which he obtained from cases of suspected dysentery and from convalescents. Of 11 strains studied by him, all fermented glucose, mannitol, and maltose readily; 6 fermented sucrose; and 2 fermented dulcitol;

in no case, with gas production. Indol was formed by 4 strains only. Litmus milk was acidified and ultimately clotted. None of the strains were agglutinated by Flexner-Y dysentery antiserum.

Thjøtta (1919), in Norway, while investigating cases of dysentery, isolated 40 strains of Flexner organisms and 25 strains of the Sonne type. He believed the Sonne cases to be of much more frequent occurrence than his figures would indicate, explaining that the less frequent finding of the Sonne type was due to the fact that this organism often produced such a mild diarrhea that the services of a physician were not deemed necessary, with the result that the cases were not subjected to bacteriological examination. Thjøtta emphasized certain characteristics of the Sonne organism that should serve for its identification, viz.: it produced large, irregular, crenated colonies when grown on litmus agar plates; acid was produced in glucose and maltose, and occasionally in lactose; no indol was formed; and serologically the group showed no relationship to the other groups of dysentery bacilli.

Mita (1921), in Japan, while studying dysentery in children, encountered bacilli similar in their cultural characteristics to those described by Sonne. Wiseman (1927) later showed by serological means that Mita's strains were identical with those of Sonne. Patterson and Williams (1922), in Australia, reported the finding of the Sonne bacillus in patients suffering from entero-colitis, dysentery, and summer diarrhea. They found that the organism on isolation required from seven to ten days for the production of acid in lactose peptone water, but that after repeated subculture the acid production occurred earlier.

Nabarro (1923) reported the isolation of late-lactose fermenting bacilli from cases of summer diarrhea and other diarrheal conditions in children. He believed the organism to be the same as a coliform bacillus isolated by Lembke, in 1896, from the excreta of dogs, to which he had given the name *B. coli-anaerogenes*. Later, Nabarro (1927) expressed the belief that *B. coli-anaerogenes* and dysentery bacilli of the Sonne type were identical, and suggested the name *B. coli-dysenteriodes* for the group.

Castellani (1927) described in some detail certain organisms isolated by him from clinically typical cases of dysentery, from cases of obscure chronic colitis with no dysenteric symptoms, and from cases in which "fever was so prominent a symptom as to suggest the possibility of paratyphoid." Most of his strains agreed in the properties of being non-motile, in not liquefying gelatin, in forming acid only in litmus milk, and in not being agglutinated by the usual dysentery anti-serums. The strains all agreed in the properties of not producing gas in any sugar, and in the production of acid in lactose, glucose, and maltose peptone water. These organisms, Castellani calls the meta-dysentery bacilli, as contrasted to the dysentery bacilli (Shiga, Shiga-Kruse) and the paradysentery bacilli (Flexner, Hiss-Russell, etc.).

Bamforth (1923) described a small outbreak of Sonne dysentery among nurses. Smith (1924) studied a small outbreak of Sonne dysentery in infants in Aberdeen. He obtained a culture of the Sonne organism from the National Collection of Type Cultures and compared this with his strains, finding them to be identical in fermentation and serological reactions. Fraser Kinlock and Smith (1926) in a study of dysentery in Aberdeen, covering a period of thirty months, encountered 6 cases of amoebic dysentery, 17 cases of Flexner dysentery, and 33 cases of Sonne dysentery. Channon (1926) isolated a strain of dysentery bacilli which she compared with a known Sonne strain, finding the two to be identical culturally and serologically.

Fyfe (1927) recorded an epidemic of 150 cases of dysentery at St. Andrews, with milk-borne *E. dysenteriae* Sonne as the causative factor.

Cases of dysentery caused by the Sonne bacillus have seldom been reported in this country, from which the inference may be drawn that in the past this type of dysentery has been quite rare in America. Recently, Gilbert and Coleman (1929) reported the occurrence of 12 cases of Sonne dysentery in New York State, 4 cases occurring in two state hospitals, 5 cases in a city during an epidemic of enteric disease attributed to a polluted water supply, and the remaining 3 cases in widely separated counties. The

identity of the causative organism was established by cultural reactions and by agglutination with an anti-serum prepared with a strain of *E. dysenteriae* Sonne obtained from the American Type Culture Collection

CLINICAL FEATURES OF SONNE DYSENTERY

Thirty-two cases of dysentery have been encountered caused by the Sonne bacillus, 29 in children and infants and 3 in adults. Of these, 5 have been of severe nature, the remainder of mild character. In the severe cases, there was one death directly attributable to the dysentery infection. In the milder cases, one death occurred, but in this case the dysentery infection was superimposed on a decompensated heart and could be considered little more than a contributing cause of death. Autopsy, unfortunately, was not permitted in this case.

Fraser, Kinlock and Smith (1926) observed 33 cases of Sonne dysentery in Aberdeen, noting a marked variation in the degree of dysenteric symptoms. They described the great majority of their cases as being characterized by a mild, subacute diarrhea associated usually with some elevation of temperature, and nearly always accompanied or preceded by a catarrhal condition of the upper respiratory system. They regarded this catarrh as a notable feature of the disease. Of the severer cases, they describe two types:

> In one variety the symptoms simulate those of an acute Flexner dysentery with sudden onset of illness, diarrhea and colic, and the rapid appearance of blood and mucus in the stools. In the second variety, the symptoms simulate those of Salmonella infections or the choleraic form of dysentery, with sudden onset, epigastric pain, vomiting, diarrhea with green stools containing mucus but no blood, and rapid prostration.

In the majority of the cases seen here there have been few or no respiratory symptoms. The illness has usually been ushered in by a temperature rise to 100° to 104°, followed after twenty-four to forty-eight hours by the passage of loose, watery, greenish-yellow stools containing mucus and sometimes blood. Blood

has not been a prominent feature in the stools of these cases, though few have been seen that at some time during the illness failed to show it. The number of stools passed has varied from 4 to 10 in a day, the diarrhea usually persisting for from two to six days, after which the stools become normal in number, but are apt to remain soft and unformed for some days. Vomiting has not occurred in most of these milder cases, but has been present in a few. Abdominal tenderness has been present in a few cases. Colic and tenesmus have been rare symptoms. The fever, in most cases, has been of short duration, twenty-four to forty-eight hours, but in a few instances has persisted for longer periods. The leukocyte count has in most cases been somewhat elevated with an increase in the polymorphonuclear cells. The following case histories will serve to illustrate the milder form of the disease, as it has been seen here:

Case 1. V. B., a nurse, on September 16 complained of diarrhea of two days duration. That night she had a fever of 103.8°, complained of chills and malaise, and passed many, small, loose, watery, yellow stools containing mucus and streaks of blood. At this time she was definitely tender over the lower part of her abdomen, but there was no spasm. The following day her temperature fell to normal, where it remained subsequently. She continued to pass loose stools at infrequent intervals for the next four days, but otherwise felt well.

Laboratory data showed a leukocyte count of 8500 on September 18, four days after the onset of her illness. Sonne dysentery bacilli were isolated from 3 stools passed on the same date. These were agglutinated by the patient's serum in a dilution of 1:80. Sonne organisms were repeatedly isolated from stool specimens examined until October 14, five weeks after the onset of her illness, after which date 3 successive negative stools were obtained.

Case 2. S. A., a white, female infant of fifteen months was admitted to the hospital on October 3, with the complaint of fever and diarrhea. The patient had been well until three days before entry when she began to refuse her feedings and seemed feverish. She then developed diarrhea, passing from 4 to 6 loose, watery stools during the day. She vomited several times before entry. On admission to the hospital, examination showed a well developed and nourished infant, very

irritable and appearing acutely ill. The temperature was 102°. The examination was otherwise negative. During the first three days in the hospital, the temperature remained at 102° and the patient passed numerous, loose, green, watery stools containing mucus and a few streaks of fresh blood. After the fourth day the temperature became normal. The child continued to pass loose stools for several days, but seemed otherwise well.

Laboratory data showed the leukocyte count on admission to be 15,600 with 74 per cent of the cells polymorphonuclears. Two weeks later the leukocyte count was 8600 with 53 per cent polymorphonuclears. *E. dysenteriae* Sonne was recovered in large numbers from a stool passed the day following admission. Subsequent stool examinations were negative. On October 8, one week after the onset of the illness, the patient's serum failed to show any agglutination with typhoid, paratyphoid A, paratyphoid B, Flexner and Hiss dysentery antigens, but agglutinated the organism recovered from the patient in a dilution of 1:320.

Case 3. R. D., a white male child of seven years eight months, was admitted to the hospital on November 16 for the correction of deformities resulting from a cord tumor. A tendon transplant operation was performed on November 22. The day following operation the temperature rose to 101° and the pulse rate to 120. The following day the patient passed 4 loose, watery, green stools containing mucus but no blood. There was no vomiting, abdominal pain, or other symptoms. The temperature varied between 99° and 101° for the next six days, during which time he continued to pass from 4 to 8 loose stools daily. After the sixth day, the temperature remained normal and the stools became normal in number and character.

E. dysenteriae Sonne was isolated from a stool passed on November 28, and from subsequent stools until December 7, when they became negative.

In the more severe cases prostration has been much more marked than in the foregoing group. Vomiting has usually been a prominent symptom, and abdominal colic and tenesmus have been commoner than in the milder cases. The following case histories illustrate the severer type of cases:

Case 4. M. W., the wife of a doctor, was taken acutely ill with vomiting, generalized abdominal pain, fever, and diarrhea on Septem-

ber 18. She had been quite well prior to the present illness and had had no similar attacks in the past. Loose stools were passed at frequent intervals, accompanied by tenesmus, the stools being as many as 15 daily. The passages were described as being watery in appearance, sometimes approaching a "rice-water" character. The patient continued to have abdominal distress, and blood appeared in the stools on the third day of the illness, at which time a stool specimen was brought to the laboratory for culture. The patient was treated symptomatically with gradual improvement, but she was forced to remain in bed for ten days.

E. dysenteriae Sonne was isolated from the stool examined, the organism being present in almost pure culture. Subsequent stools were not obtained for culture.

Case 5. M. M. (2), a white, female child of three and one-half years was admitted to the hospital on October 25 because of persistent vomiting, fever, and prostration. The past history revealed that since one and one-half years of age, she had had attacks of vomiting at regular intervals until the previous spring, since which time she had been free of symptoms. Thirty hours before entry, she began vomiting and had continued to display this symptom, together with feverishness and great prostration. There had been no diarrhea noted. Examination showed a well developed and nourished child with flushed cheeks, an apprehensive expression, and considerable evidence of dehydration. The breathing was rapid, shallow, and irregular, and there was an acetone odor to the breath. The tonsils were moderately large and both ear drums were thought to be injected. She was thought to have an acute upper respiratory infection with profound ketosis, though the condition of the throat and ears did not seem sufficient to account for the extreme prostration and a temperature of 105°, which was present. Examination of the urine showed a trace of acetone, but was otherwise negative. Both ear drums were incised without relief. Energetic treatment was instituted to combat the acidosis and dehydration, but the symptoms continued unabated. On the 28th, three days after admission to the hospital, the patient suddenly became comatose and respirations ceased a few hours later.

Post-mortem examination showed evidence of marked dehydration, a fatty liver, and engorgement of the esophageal and gastric mucosae. There were numerous hemorrhagic areas in the ilium and jejunum and swollen mesenteric nodes, suggesting an enteric infection. Micro-

scopic section through these hemorrhagic areas showed marked diphtheritic inflammation of the mucosa with partial loss of the mucosal surface, fibrin deposit, and polymorphonuclear cell infiltration.

Cultures taken at autopsy from both the intestinal contents and the right ureter grew out colonies of *E. dysenteriae* Sonne in large numbers.

Case 6. P. M., a white, male child of three and one-half years, was admitted to the hospital January 1st, with the complaint of convulsions. The family history revealed that one week before the onset of the patient's illness, a sibling, aged forty-four days, had died suddenly of unknown cause, and that four days before, another sibling, aged nineteen months, had recovered from an attack of vomiting and diarrhea. The patient had been well until the night before entry, when he vomited and developed diarrhea, passing 8 loose stools containing mucus. The morning of the day of admission the diarrhea continued and he had two generalized convulsions, each lasting about fifteen minutes. On admission the temperature was 103°. Examination showed a well developed and nourished child appearing acutely ill. The throat was injected and there was evidence of marked dehydration and acidosis. Otherwise the examination was negative. Intravenous glucose and subcutaneous salt solution were given immediately with rapid improvement. The following day the temperature was normal and the patient seemed otherwise well, though he continued to pass an increased number of stools containing mucus for about a week.

Laboratory data showed the leukocyte count to be 8150 with 84 per cent polymorphonuclear cells. Cultures of the stools on the day of admission yielded many colonies of *E. dysenteriae* Sonne. Stool cultures remained persistently positive up to February 10, when the first negative culture was obtained. An attempt was made on February 13 to culture the organisms from aspirated bile and duodenal contents. The attempt was not successful. The patient's serum on February 15, six and one-half weeks after the onset of the illness, failed to agglutinate Flexner or Hiss dysentery organisms, but agglutinated Sonne organisms in a dilution of 1:40. The patient was discharged from the hospital February 18, remaining, however, under the observance of the Board of Health.

From the foregoing description of the disease, it is seen that dysentery caused by the Sonne bacillus may be either quite mild or of a very alarming nature. While the great majority of the cases are not severe enough to cause any great concern, occa-

sionally one encounters a case that, without careful bacteriological study, could not be distinguished from some of the more severe types of enteric disease. That the Sonne bacillus is possessed of considerable invasive power is shown by the autopsy findings in case 5. It has been noticed that the organisms are apt to remain in the feces for weeks after the subsidence of the symptoms, and for this reason carriers are probably quite common and important in the transmission of the disease. The persistence of the organisms in the stools of convalescents is well shown in cases 1 and 6.

CULTURAL CHARACTERISTICS

In the isolation and identification of *E. dysenteriae* Sonne the same technique has been employed as with other members of the typhoid-dysentery group of organisms, which in brief is as follows:

As soon after passage as feasible, the stool specimen is brought to the laboratory, where it is plated out on an eosin-methylene-blue agar plate. After twenty-four hours incubation, suspicious colonies are fished to Russell's double sugar agar slants. Organisms giving a suspicious reaction on Russell's medium are then tested for motility, run through fermentation reactions, and later agglutinated with appropriate immune serums. For the routine fermentation tests, we have employed 8 sugars: glucose, mannitol, maltose, lactose, xylose, rhamnose, sucrose, and dulcitol. Motility is examined for, using twenty-four-hour broth cultures grown at room temperature.

E. dysenteriae Sonne has been found to differ but little morphologically from other dysentery organisms. The organism is a short, coliform bacillus showing a tendency toward involution forms. It is decolorized by Gram's method of staining. It is non-motile. It does not liquify gelatin, even after twenty-one days cultivation on this medium. Indol is not produced. Litmus milk is rendered acid after twenty-four hours, but clotting does not occur even after twenty-eight days, the milk remaining acid throughout this period.

On eosin-methylene-blue agar plates, the Sonne organism produces round, well defined, greyish-white to clear colonies, indistinguishable from the colonies of other dysentery bacilli

after twenty-four hours incubation. However, after longer incubation, the colonies become larger, more irregular in outline, and present a more or less characteristic crenated border. On plain hormone agar and in liquid media the growth is not different from that of other members of the typhoid-dysentery group of organisms.

In contradistinction to the results reported by certain other workers, the Sonne bacillus has been found to be fairly uniform in its fermentation reactions. Twenty-eight strains of the organism recovered from 28 cases of dysentery have been examined in detail. Slight cultural variations have been found within the group (see table 1). The fermentation reactions are as follow:

Glucose: Uniform acid production without gas in twenty-four hours.

Mannitol: Uniform acid production without gas in twenty-four hours.

Maltose: Uniform acid production without gas in twenty-four hours.

Lactose: Acid production without gas, usually apparent by the third day, but occasionally delayed as long as the seventh day.

Xylose: No acid or gas, even after twenty-one days.

Rhamnose: Uniform acid production without gas in twenty-four hours.

Sucrose: Delayed acid production without gas was noted with 15 of 28 strains. Acid production, when present, was usually apparent by the sixth day, but in one instance was not evident until the fifteenth day. Thirteen strains failed to produce acid, though incubated for twenty-one days.

Dulcitol: Acid or gas not produced, even after twenty-one days.

Glycerol: Delayed acid production without gas was noted with 14 of 28 strains. Acid production, when present, was usually noted about the twelfth day, but in one instance did not appear until the twenty-first day. Fourteen strains failed to produce acid, though incubated for twenty-one days.

Inulin: Neither acid nor gas produced, even after twenty-one days.

Dextrin: A slight production of acid occurred with 17 of 28 strains, usually present after twenty-four hours incubation, but in one instance delayed until the tenth day. Eleven strains failed to produce acid, even after twenty-one days.

Sorbitol: Acid or gas not produced, even after twenty-one days.

TABLE 1

STRAIN	MOTILITY	GELATIN	INDOL	LITMUS MILK	GLUCOSE	MANNITOL	MALTOSE	LACTOSE	XYLOSE	RHAMNOSE	SUCROSE	DULCITOL	GLYCERINOSE	INULIN	DEXTRIN	SORBITOL	GALACTOSE	HAFFINOSE	ARABINOSE	LEVULOSE	SALICIN	INOSITE
G. R.*	—	0	0	A	A	A	A	—	—	A	—	—	—	—	—	—	A	A(3)	A	A	—	—
D. D.*	—	0	0	A	A	A	A	—	—	A	—	—	—	—	—	—	A	A(4)	A	A	—	—
L. M.*	—	0	0	A	A	A	A	—	—	A	—	—	—	—	—	—	A	A(2)	A	A	—	—
T. F.*	—	0	0	A	A	A	A	—	—	A	—	—	—	—	—	—	A	A(6)	A	A	—	—
V. B.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	—	—	A	A(4)	A	A	—	—
M. W.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	—	—	A	A(3)	A	A	—	—
S. A.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	—	—	A	A(3)	A	A	—	—
J. B.	—	0	0	A	A	A	A	A(6)	—	A	—	—	—	—	—	—	A	A(10)	A	A	—	—
M. M. 1.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	—	—	A	A(4)	A	A	—	—
M. M. 2.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	—	—	A	A(3)	A	A	—	—
E. N.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	—	—	A	A(3)	A	A	—	—
H. K.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	AS(5)	—	A	A(10)	A	A	—	—
R. N.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	AS(3)	—	A	A(4)	A	A	—	—
M. L.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	—	—	A	A(6)	A	A	—	—
R. D.	—	0	0	A	A	A	A	A(3)	—	A	—	—	—	—	AS(10)	—	A	A(4)	A	A	—	—
H. R.	—	0	0	A	A	A	A	A(4)	—	A	—	—	—	—	—	—	A	A(3)	A	A	—	—
G. N.	—	0	0	A	A	A	A	A(3)	—	A	A(15)	—	—	—	—	—	A	A(6)	A	A	—	—
P. M.	—	0	0	A	A	A	A	A(6)	—	A	—	—	—	—	—	—	A	A(4)	A	A	—	—
L. H.	—	0	0	A	A	A	A	A(3)	—	A	A(6)	—	A(9)	—	AS	—	A	A(5)	A	A	—	—
C. B.	—	0	0	A	A	A	A	A(5)	—	A	A(6)	—	A(17)	—	AS	—	A	A(2)	A	A	—	—
B. S.	—	0	0	A	A	A	A	A(3)	—	A	A(6)	—	A(8)	—	AS	—	A	A(5)	A	A	—	—
L. F.	—	0	0	A	A	A	A	A(2)	—	A	A(6)	—	A(10)	—	AS	—	A	A(3)	A	A	—	—
N. M.	—	0	0	A	A	A	A	A(3)	—	A	A(12)	—	A(21)	—	AS	—	A	A(2)	A	A	—	—
F. J.	—	0	0	A	A	A	A	A(4)	—	A	A(6)	—	A(12)	—	AS	—	A	A(3)	A	A	—	—
R. M.	—	0	0	A	A	A	A	A(3)	—	A	A(6)	—	A(10)	—	AS	—	A	A(2)	A	A	—	—

N. S.	–	0	0	A	A	A	A	A(7)	–	A	A(6)	–	A(16)	–	AS	–	A	A(2)	A	A	–	–
M. C.	–	0	0	A	A	A	A	A(3)	–	A	A(6)	–	A(10)	–	AS	–	A	A(3)	A	A	–	–
S. L.	–	0	0	A	A	A	A	A(2)	–	A	A(2)	–	A(12)	–	AS	–	A	A(2)	A	A	–	–
E. A.	–	0	0	A	A	A	A	A	–	A	A(6)	–	A(12)	–	AS	–	A	A(5)	A	A	–	–
A. M.	–	0	0	A	A	A	A	A(3)	–	A	A(6)	–	A(16)	–	AS	–	A	A(3)	A	A	–	–
J. M.	–	0	0	A	A	A	A	A(4)	–	A	A(6)	–	A(7)	–	AS	–	A	A(3)	A	A	–	–
D. A.	–	0	0	A	A	A	A	A(7)	–	A	A(6)	–	A(17)	–	AS	–	A	A(3)	A	A	–	–

Symbols: (–) No acid or gas; (A) Acid only; [A(4)] Acid formed after 4 days; (AS) Slight acid formation.
* Eight sugars only—twenty-four hours incubation only.

TABLE 2

PATIENT	PATIENT'S ORGANISM VS. ANTISERUM						PATIENT'S SERUM VS. ORGANISM			
	Flexner	Hiss	Polyvalent dysentery	Sonne	"M.W.-r"	Miscellaneous convales.	Flexner	Hiss	Patient's organism	Miscellaneous
G. R.	0	0	0				0	0	1:160	
D. D.	0		0				0	0		
L. M.	0		0							
T. F.	0	0	0			VB 1:80 SA 1:160	0	0	0	
V. B.	0	0	0	1:5120	1:2560	SA 1:160	0	1:20	1:80	MW 1:80 TF 1:80 SA 1:80
M. W.	0	0	0	1:5120	1:5120	VB 1:80 SA 1:160				
S. A.	0		0	1:2560	1:2560	VB 1:80	0	0	1:320	JB 1:320; VB 1:160 MW 1:160; MM 1:320
J. B.	0		0	1:2560	1:2560	SA 1:320				
M. M. 1.	0	0	0	1:5120	1:5120	SA 1:320				
M. M. 2.	0		0	1:2560	1:2560	SA 1:320	0	0	1:320	RD 1:320; HR 1:320 GN 1:320; PM 1:320
E. N.	0		0	1:5120	1:5120	SA 1:320	0	0	0	
H. K.	0		0	1:2560	1:2560	SA 1:320				
R. N.	0		0	1:2560	1:2560	SA 1:320				
M. L.	0		0	1:5120	1:2560	SA 1:320				
R. D.	0		0	1:5120	1:5120	MM(2) 1:320				
H. R.	0		0	1:5120	1:2560	MM(2) 1:320				
G. N.	0	0	0	1:5120	1:5120	MM(2) 1:320				
P. M.	0	0	0	1:5120	1:2560	MM(2) 1:320	0	0	1:40	VB 1:40

L. H.	0	0	0	1:2560	1:2560	"VB-r"* 1:320	1:40	0	1:320	VB 1:320
C. B.	0		0	1:5120	1:2560	"VB-r" 1:320	1:20	1:20	1:40	VB 1:40
B. S.	0	0	1:20	1:5120	1:5120	"VB-r" 1:320	0	0	1:40	VB 1:40
L. F.	0		0	1:5120	1:5120	"VB-r" 1:640	0	0	1:160	VB 1:40
N. M.	0	0	0	1:2560	1:2560	"VB-r" 1:320				
F. J.	0		0	1:2560	1:1280	"VB-r" 1:640				
R. M.	0		0	1:5120	1:2560	"VB-r" 1:320				
N. S.	0		0	1:5120	1:2560	"VB-r" 1:320	0	0	1:20	VB 1:20
M. C.	0		0	1:2560	1:2560	"VB-r" 1:320	0	0	1:40	VB 1:20
S. L.	0	0	0	1:5120	1:5120	"VB-r" 1:80	1:20	1:20	1:40	VB 1:40
E. A.	0		1:20	1:5120	1:5120	"VB-r" 1:640	0		1:160	VB 1:40
A. M.	0		0	1:2560	1:2560	"VB-r" 1:640	0		1:160	VB 1:20
J. M.	0		0	1:2560	1:5120	"VB-r" 1:320	1:20		0	VB 0
D. A.	0		0	1:5120	1:5120	"VB-r" 1:320	1:20		0	VB 0

"M. W.-r" = immune serum from rabbit immunized with strain MW (titer 1:5120).

* "VB-r" = first immune serum from rabbit immunized with strain VB (titer 1:640).

Galactose: Uniform acid production without gas in twenty-four hours.

Raffinose: Uniform acid production without gas, usually present in two to six days, but in one instance delayed until the tenth day.

Arabinose: Uniform acid production without gas in twenty-four hours.

Levulose: Uniform acid production without gas in twenty-four hours.

Salicin: Neither acid nor gas produced, even after twenty-one days.

Inositol: Neither acid nor gas produced, even after twenty-one days.

SEROLOGICAL REACTIONS

The majority of these cases of Sonne dysentery occurred before either Sonne immune-serum or a known culture of *E. dysenteriae* Sonne was available. However, it was early recognized that this was a type of dysentery caused by an organism that had not been previously encountered here. Each strain was tested, when isolated, for agglutinability with Flexner dysentery anti-serum (Lederle) and polyvalent dysentery anti-serum (Mulford), with uniformly negative results. That the strains were identical, was proved by cross agglutination reactions obtained by using at first patient's immune-serum and later immune serum from rabbits inoculated with emulsions of strains VB (VB-r serum, titer 1:640) and MW (MW-r serum, titer 1:5120). The results of these cross agglutination tests are given in table 2.

A COMPARISON OF E. DYSENTERIAE SONNE WITH CERTAIN OTHER LATE-LACTOSE FERMENTING BACILLI

Noting the similarity existing between *E. dysenteriae* Sonne, the metadysentery bacilli described by Castellani (1927), and *E. dispar* described by Andrewes (1918), cultures of these organisms were obtained from the American Type Culture Collection and were subjected to cultural and serological comparison. Immune serums were prepared in rabbits and cross agglutination reactions carried out. The three organisms have been found to be identical in morphology, in being non-motile, in not liquefying gelatin, in fermenting lactose late, and in rendering milk acid without clot formation. In their fermentation reactions, *E.*

dysenteriae Sonne (*Eberthella paradysenteriae* Sonne no. 31) and the metadysentery bacillus (*E. metadysenteriodes* no. 4086) have been practically identical, while *E. dispar* (*Eberthella dispar* no. 29) has been distinctly different in this regard. *E. dispar* has also differed from the other two organisms in producing indol. These results are shown in table 3. *E. dysenteriae* Sonne and *E. meta-*

TABLE 3

ORGANISM	MOTILITY	GELATIN	INDOL	LITMUS MILK	GLUCOSE	MANNITOL	MALTOSE	LACTOSE	XYLOSE	RHAMNOSE	SUCROSE	DULCITOL	GLYCEROL	INULIN	DEXTRIN	SORBITOL	GALACTOSE	RAFFINOSE	ARABINOSE	LEVULOSE	SALICIN	INOSITE
Eberthella paradysenteria Sonne	–	0	0	A	A	A	A	A (2)	–	A	A (5)	–	–	–	A (3)	–	A	A (3)	A	A	–	–
E. metadysenteriodes Castellani	–	0	0	A	A	A	A	A (3)	–	A	A (5)	–	A (3)	–	–	–	A	A (4)	A	A	–	–
Eberthella dispar Andrewes	–	0	+	A	A	A	A	A (2)	A	A	A	–	A	–	AS	A	A	A (6)	A	A	–	–

TABLE 4

ORGANISMS	IMMUNE SERUM		
	Sonne dysentery	Castellani metadysentery	Andrewes *E. dispar*
	Titer		
	1:5120	1:5120	1:5120
Eberthella paradysenteriae Sonne	1:5120	1:5120	0
E. metadysenteriodes (Castellani)	1:5120	1:5120	0
E. dispar (Andrewes)	0	0	1:5120

dysenteriodes have been agglutinated equally well in immune serums prepared with each of these organisms, while *E. dispar* has not been agglutinated by these serums. Likewise, an immune serum prepared with *E. dispar* failed to agglutinate *E. dysenteriae* Sonne or *E. metadysenteriodes*, but agglutinated the homologous organism in a dilution of 1:5120. These cross agglutination results are given in table 4.

It may be concluded that *E. dysenteriae* Sonne and *E. dysenteriodes* Castellani are strains of the same organism and should not be separately classified, while *E. dispar* Andrewes differs distinctly from these, and consequently is entitled to a place of it own in the late-lactose fermenting group of organisms.

DISCUSSION

Dysentery caused by the Sonne bacillus has been reported in Norway, France, Japan, Australia, England, and Scotland, but until very recently has not been encountered on this continent. The finding of 32 cases of this type of dysentery in Boston, would indicate that the disease may have a more than casual incidence.

Most cases of dysentery caused by the Sonne bacillus are of a mild diarrheal nature, but from time to time more severe cases occur in which the picture may closely simulate a Salmonella infection. The importance of careful bacteriological study is apparent, both from a clinical and epidemiological aspect.

Dysentery of the Sonne type is not difficult of recognition bacteriologically, the outstanding characteristics of the causative organism being its cultural similarity to the Flexner bacillus, its latent formation of acid in lactose containing media, its inagglutinability in Flexner dysentery anti-serum, and its agglutinability with Sonne dysentery anti-serum.

SUMMARY

Thirty-two cases of dysentery caused by *Eberthella dusenteriae* Sonne are reported.

Clinical features of the disease and case histories illustrating the mild and severe forms are given.

A descriptive of the causative organism and its reactions is given in detail.

The metadysentery bacilli described by Castellani are shown to be identical with *E. dysenteriae* Sonne, while *E. dispar* described by Andrewes is shown to be entirely different from these organisms.

Attention is drawn to the fact that *E. dysenteriae* Sonne is important as a cause of dysentery in this country, as indicated by this study.

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A METHOD OF STAINING BACTERIAL FLAGELLA AND CAPSULES TOGETHER WITH A STUDY OF THE ORIGIN OF FLAGELLA

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BACTERIAL FLAGELLA

Flagella staining is generally considered a rather difficult procedure and even with the most careful technique only a few of a great number of slides show satisfactory flagella. The main trouble with most flagella stains is that the mordant which is used is not stable. Good results may be obtained at one time and poor results the next. An important property of a good flagella stain should, then, be a stable mordant. Such a mordant was described by Gray (1). He mixed potassium alum, mercuric chloride, tannic acid, and alcoholic fuchsin and immediately poured the mixture on the smear. All these reagents are perfectly stable and the stain can always be duplicated. It is excellent as compared with other flagella stains. A counter stain of a strong solution of basic fuchsin is also necessary. This stain gives most excellent results with the sporulating organisms such as *B. vulgatus*, *B. subtilis*, *B. terminalis*, *Cl. botulinum*, *Cl. tetani*, etc. With *Bact. typhosum*, and the Vibrios, not so good results are obtained. Various modifications of the stain were tried. A distinct improvement was the addition of sufficient hydrochloric acid to the alum to make the concentration of acid 1/100 N. Ammonium alum in place of potassium alum was found to give fully a good result. However, it was not until addition of alcohol was tried that an almost perfect flagella stain was obtained. Without the alcohol in the so-called "mordant," the flagella can be seen very faintly without the counter stain. With

the alcohol, no counter stain is needed, and the flagella are intensely stained and enormously enlarged.

METHOD OF MAKING SMEARS

The slides

The slides must be as clean as possible. Our method is to put the old slides in an alkaline soap solution to remove the oil, then into good cleaning solution for a day or so. The slides are then washed carefully with distilled water and dried between paper towels. Washing with alcohol seems to have no additional cleaning effect. The slides selected should be as free from scratches as possible. Just before use they are flamed strongly.

The organisms

The organisms are best grown in bouillon for twelve to twenty-four hours from which they are centrifuged, suspended in distilled water, recentrifuged, and resuspended to give a slightly milky suspension. This amount of washing is usually sufficient but further washing may sometimes give a clearer slide. Very good results may also be obtained by making a dilute suspension of the organisms from an agar slant. A loopful of the organisms is placed on the end of the slightly warm slide and allowed to run down the slide. If the water does not run down the slide it is greasy and should be discarded. Contrary to general opinion the flagella on some bacteria at least, are not easily broken off. A suspension of typhoid bacilli in distilled water was shaken in a shaking machine for twenty minutes with only a loss of about 50 per cent of the flagella. Suspended in distilled water, the flagella on typhoid bacilli were found to remain intact for one month. No stains were made after this length of time. *Bacillus terminalis* flagella were still intact on the organisms after two weeks in distilled water.

The stain

Basic fuchsin is by no means the only dye which can be used in the flagella stain. Methylene blue and crystal violet have been

used with about equal success. Differences in the solubilities of these dyes in alcohol makes it necessary to add them in different amounts to obtain the best results. With basic fuchsin the stain has the following composition:

	cc.
Ammonium or potassium alum, saturated water solution	20
Tannic acid, 20 per cent water solution	10
Distilled water	10
95 per cent ethyl alcohol	15
Saturated ethyl alcoholic solution of basic fuchsin	3

Crystal violet is about twice as soluble in alcohol as is basic fuchsin. If this dye is used, only about 1.5 cc. of it should be added in place of 3 cc. of basic fuchsin. Methylene blue is soluble to only the amount of about 1.5 per cent, or one-fourth the solubility of basic fuchsin. Since considerable alcohol is thus introduced with the dye the amount of alcohol added as such should be reduced to one-half. With methylene blue the following formula is recommended:

	cc.
Ammonium or potassium alum, saturated water solution	20
Tannic acid, 20 per cent water solution	10
Distilled water	5
95 per cent ethyl alcohol	5
Saturated alcoholic solution of methylene blue	10

The ingredients are mixed in the order given. A slight precipitate usually forms which may be filtered off but this is not necessary. The stain should be kept in a tightly stoppered bottle and will keep for a week or more. As the stain gets old it stains more faintly than at first but less precipitate also forms on the slides. The stain will remain in good condition longer if a greater amount of dye is added, but only after one or more days does it really become good. If the stain is to be used at the time of preparation only, the quantities of dye which are added may be reduced. Instead of 3 cc. of fuchsin 2 cc. will give somewhat better results.

On a warm day the unfixed smears may be flooded with the stain and left at room temperature for ten minutes. On a cold day, however, it is better to place them in the incubator. The

higher the temperature the more deeply stained the flagella will be but more precipitate will also form on the slide. After about ten minutes the stain is washed off with water.

Counter stain

As far as the flagella themselves are concerned no counter stain is needed. Very beautiful preparations can be obtained, however, by using a counter stain of a color different from that of the stain. With the flagella stained red the organisms can be stained blue by counter staining for five to ten minutes with an aqueous solution of methylene blue made as follows:

	per cent
Methylene blue (dry powder)	0.1
Borax	1

Carbol fuchsin diluted 1/10 with water applied for five to ten minutes makes a good counter stain in case the flagella are blue.

DISCUSSION

Very little work seems to have been done lately on the nature of bacterial flagella. The most complete discussion of the subject seems to be that of Migula in his well-known "System der Bakterien." From the data then at hand Migula concluded that flagella were out-growths of the bacterial ectoplasm. This idea also seems to be generally accepted at the present time. From the appearance of flagellar preparations of *Bact. typhosum*, and *Proteus vulgaris* a different conclusion may be drawn. As already stated, neither of these organisms stains solidly with the flagella stain. The flagella, the ectoplasmic zone, and a central column are stained red. The rest of the organism is colorless unless a counter stain is used in which case it takes the color of the counter stain. In a number of cases the flagella are seen to originate in the central column. Only rarely does a flagellum originate between the central column and the ectoplasmic zone. Most frequently the flagella seem to originate on the surface. Figure 1 shows drawings of a number of typhoid bacilli. Figure 2 is a

photograph of typhoid bacilli. It is possible that the central column is an artefact, and represents a shrunken endoplasm. This being the case the clear zone between it and the ectoplasm should be empty. This would not seem to be the case, however,

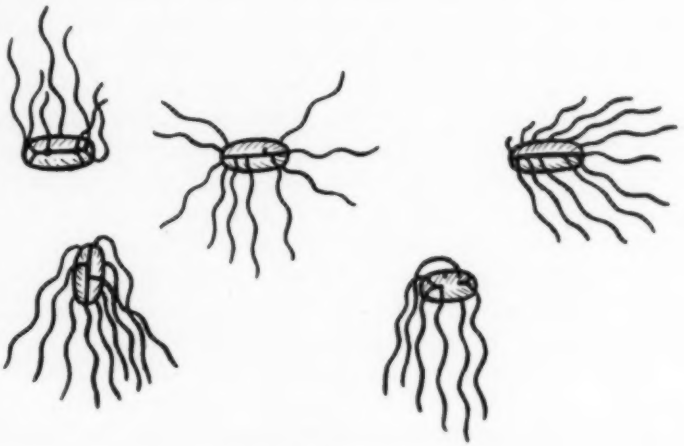

FIG. 1. BACTERIUM TYPHOSUM

FIG. 3. VIBRIO FINKLOR-PRIOR

from the way it takes the counter stain. Even very dilute alkaline methylene blue stains it quite heavily. The alternative explanation would be that the central column is a definite structure running along the centre of the organism and having a composition similar to that of the flagella.

The sporulating organisms studied, both Gram positive and Gram negative, stain solidly except in the case of *B. cereus*. In the case of this organism the ectoplasm is stained only in patches. The vibrios (*V. metchnikovi*, and *V. finklor-prior*) stain evenly and somewhat faintly. When counter stained with Manson's methylene blue (5 per cent borax + 1 per cent methylene blue) many of them show the interesting structure illustrated in figure 3.

From the data obtained, with *Bact. typhosum* especially, it might be concluded that bacterial flagella are not of ectoplasmic origin but originate in the endoplasm, and perhaps in come definite structure in the endoplasm. This conclusion would find substantiation in the work of Smith and Reagh (2) where it was shown that agglutinins produced against a motile strain of Hog-cholera organisms were not very active against a non-motile strain of the same organism. It is concluded that the agglutinins for the flagella and the cell body are different.

In the case of organisms having terminal spores, the flagella as a rule first fall off the end in which the spore is located. This perhaps may not be taken to indicate that the flagella come from the endoplasm but merely that the ectoplasm is weakened around the spore. Figures 4 and 5 are photographs of *Cl. botulinum* showing both flagella and spores. Figure 4 was especially selected to show an organism in which the flagella were still intact even on the spore end.

Flagella and capsules may be present on the same organisms. An especially beautiful demonstration of this is found in the case of *Ps. aeruginosa* (pyocyaneus) as shown in figure 6. These cells were obtained at autopsy from the peritoneal fluid of a guinea pig injected with the specific organism.

In the experiments on the effect of additions of acid (hydrochloric) to the flagella stain it was noticed that when a certain concentration (1/10 N) was reached the flagella would no longer stain evenly but as a series of dots. The dots are round and evenly spaced. If the acid concentration is increased still further the flagella do not stain at all. If carbol fuchsin is added to a hanging drop of Vibrios (and other organisms as well) some flagella can be seen. These flagella are not stained solidly but

as a series of dots. These dots may be artefacts, of course. If they represent real structures the probability would be from their staining behavior that they are nuclear in nature.

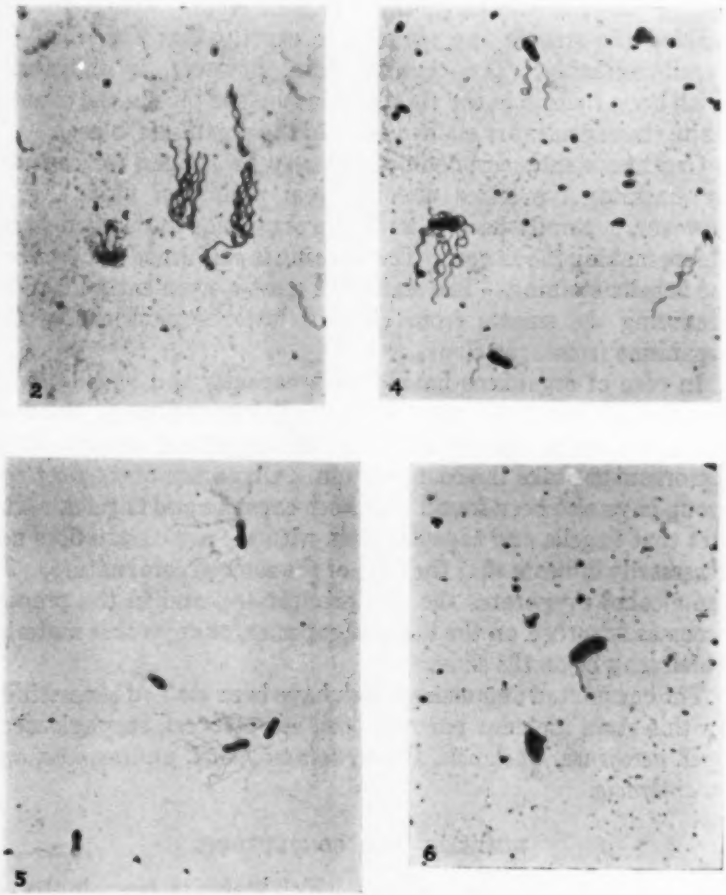

FIG. 2. BACTERIUM TYPHOSUM
FIG. 4. CLOSTRIDIUM BOTULINUM
FIG. 5. CLOSTRIDIUM BOTULINUM
FIG. 6. PSEUDOMONAS AERUGINOSA
(Magnification 1000 ×)

BACTERIAL CAPSULES

The stains generally used for bacterial capsules are not altogether satisfactory. The stain for flagella described in the preceding pages is also an excellent capsule stain. The method of making the smears and staining is exactly that described for flagella staining. The counter stain, however, is obligatory. With basic fuchsin in the stain and methylene blue in the counter stain, the capsules are stained red and the organisms blue.

Organisms such as pneumococci may be stained for capsules by smearing the slides directly from peritoneal fluid. It is, however, generally best to wash the organisms in distilled water before making the smear. The washing is performed as described for flagella staining. The stains are most conveniently made by preparing the smears from distilled water suspensions of the organisms from agar slants.

In case of organisms having both capsules and flagella, both are stained simultaneously as illustrated by figure 6. In such cases both the capsules and flagella take the color of the stain while the organisms take the counter stain. Organisms of the *Bact. coli* group have also been found with both capsules and flagella. The fact that flagella and capsules stain with the same stain does not necessarily indicate that they are of the same general nature. As the alcohol evaporates the dye precipitates, and in the process becomes adsorbed on the flagella, capsules, or any other material which may be on the slide.

The capsulated organisms which have been stained successfully by this stain include: pneumococci, streptococci, staphylococci, *Bact. aerogenes, Bact. coli, Ps. aeruginosa, Bact. pneumoniae, and B. anthracis.*

SUMMARY AND CONCLUSIONS

1. A flagella stain is described which stains in ten minutes, is simple to make, stable for a week or more, and with which no counter stain is needed.

2. The flagella and cell bodies may be stained a different color by the use of an appropriate counter stain.

3. Evidence is produced to show that flagella may not be of ectoplasmic origin, but may originate somewhere in the endoplasm.

4. Sporulating organisms may retain their flagella although fully sporulated. The flagella fall off first where the spore is formed.

5. Flagella and capsules may be co-existent.

6. There are some indications that flagella are not homogeneous but contain masses of nuclear material distributed at regular intervals.

7. The flagella stain described is also an excellent capsule stain. This does not indicate that flagella and capsules are of similar composition.

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SOME PROPERTIES OF POLIOMYELITIS VIRUS[1]

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In view of the general lack of confidence either in the "globoid bodies" of Flexner and Noguchi or the streptococci of Rosenow as the specific etiologic agents in poliomyelitis, it would seem that some progress might be made by an indirect attack, such as that offered by the possibility of concentrating the virus by distillation in vacuo, or by some method of chemical precipitation. Either of the organisms mentioned, if of etiologic significance, must be present in the "filtrate" which is so uniformly infective. If, then, we can concentrate 50 or 100 cc. of this filtrate to 1 cc., the relative increase in the number of organisms should make them more easily cultivable (Bloomfield and Felty, 1924) and more easily demonstrable by direct microscopic examination. Also, the confusing extraneous material present in a brain cord suspension should be reduced by this procedure, and such a "concentrate" might be more effective as an artificial immunizing agent than the suspensions used heretofore.

In the summer of 1912, Flexner, Clark and Frazer employed the method of distillation in vacuo at relatively low temperatures, 20° to 30°C., for the purpose of concentrating filtered washings from the nasopharynges of "contacts" with cases of poliomyelitis. That this method did not seriously injure the virus which might be present in the throats, was shown by subjecting the regular salt solution poliomyelitis filtrate to the same procedure. Monkeys injected with brain-cord filtrate which had been concentrated

[1] This work was made possible through aid received from the International Infantile Paralysis Committee from funds given by Jeremiah Milbank.

about four or five times by volume, came down typically with characteristic symptoms and lesions. The results with the concentrated nasal washings were negative, however, and since a "carrier" of poliomyelitis virus (Flexner, Clark and Frazer, 1913) was successfully demonstrated by injecting small amounts of the original washings into the sheath of the sciatic nerves and large volumes (140 cc.) intraperitoneally, this *in vacuo* method was not published at the time.

Subsequently, Amoss and Taylor (1917) used this procedure in concentrating distilled water nasal washings from normal persons and from cases of poliomyelitis, demonstrating therewith that these washings, especially those from normal individuals, frequently had the power of inactivating poliomyelitis virus.

The method of distillation *in vacuo* hardly needs description save to mention one or two minor details which we have found helpful in applying the method to this material. Many sealed capillaries in the spherical flask containing the virus to be distilled prevented excessive bumping. Circulating ice water in the distilling tubes aided in maintaining low temperature during the warmer months; two smaller flasks connected in series with the distilling flask, and each separated by an air tight valve made it possible to add an indefinite amount of material without breaking the vacuum. Thus the process could be run continuously until the desired concentration was effected. By using new rubber fittings every two or three times and paraffin on the joints, a vacuum of 12 to 15 mm. of mercury was obtained with the water pump. For the rapid progress of the concentration, it is necessary to apply the heat rather continuously, so a double jacketed water bath with an electric heater and thermostatic control was employed. The temperature outside the virus flask has been kept at about 40° while the inside temperature has remained usually at 20° to 21°C., never going above 25°C. The whole apparatus is assembled and sterilized in the autoclave before using. It is readily possible to concentrate 500 to 600 cc. of the virus filtrate to one-fiftieth or one-sixtieth of the original volume in four to five hours.

CONCENTRATION OF THE VIRUS BY DISTILLATION IN VACUO

As we intended to concentrate the virus to a greater degree than in 1912, we at first employed in different experiments, distilled water, twentieth physiological saline or tenth physiologic saline as the diluent for the brain and cord suspension. This was to avoid possible injury to the virus through the marked increase in sodium chloride content coincident with the decrease in volume of water. We succeeded in obtaining active filtrates with these diluents, but the small amount of electrolyte in the suspensions caused such persistent turbidity, as to reduce the amount of virus that would pass through a Berkefeld or Mandler Filter as compared with that which would pass when physiologic saline was employed (see table 1, series 1). It was possible, however, to demonstrate that a filtrate made from a 10 per cent suspension of glycerinated poliomyelitis brain and cord diluted with twentieth physiologic saline could be reduced in volume by distillation in vacuo to one thirtieth of the original volume and produce the disease when injected in the amount of 0.03 cc. The control animal died of colitis so that no quantitative statement can be made with reference to this series (see table 1, series 2).

Because, however, of the relative lack of potency of the filtrates made with low salt concentrations, all of the later experiments were carried out with physiologic saline (0.85 per cent). Series 3 (table 1) shows the results of such a reduction in volume in which the "concentrate" was at least five or six times as potent as the filtrate and apparently as potent as the original brain cord suspension. The "concentrate" obtained by reducing the water content to one-thirtieth or less of the original volume is a light, reddish brown, thick, viscous fluid from which a small amount of precipitate gradually settles out leaving a moderately clear light amber colored fluid.

In the fourth series, the concentration procedures gave a residue even more potent than the original 5 per cent brain cord suspension (see table 1, series 4).

The results have not in every instance been successful, however, especially in the last six months when the virus seems to have

TABLE 1

Concentration of virus by distillation in vacuo

Injections in all cases were made intracerebrally unless otherwise stated.
Ether anesthesia was employed in making these injections.
Observations were continued for one month before concluding any experiment.
Clinical observations were controlled by microscopic examination of tissues.

SERIES	MONKEY NUMBER	SOURCE OF MATERIAL	INOCULUM	AMOUNT INJECTED	DATE OF INOCULATION	SUBSEQUENT COURSE	PREVIOUS HISTORY
1	19	14, 15	Filtrate of 10 per cent suspension in 20th physiologic saline	2 cc.	27-XII-28	Remained normal	None
	20	14, 15	Filtrate of 10 per cent suspension in physiologic saline	2 cc.	27-XII-28	Paralyzed 8-I-29 Prostrate 11-I-29 Killed 12-I-29	None
2	18	14	10 per cent filtrate in 20th physiologic saline	1 cc.	14-XII-28	Died—colitis 22-XII-28	None
	16	14	30-1 concentrate of filtrate	0.1 cc. in 1.5 cc. volume	14-XII-28	Paralyzed 8-I-29 Recovery by 1-III-29	None
	17	14	30-1 concentrate of filtrate	0.033 cc. in 1 cc. volume	14-XII-28	Paralyzed 7-I-29 Died 8-I-29	None
3	56	42, 73	Filtrate of 5 per cent suspension	2 cc.	12-XII-29	Paralyzed 20-XII-29 Prostrate 21-XII-29 Died 22-XII-29	Attempted immunization with obvious failure
	86	42, 73	Filtrate of 5 per cent suspension	1 cc.	12-XII-29	No Poliomyelitis. Died of pneumonia 11-I-30	None

	70	42, 73	90–1 concentrate of filtrate	0.15 cc. in 1.5 cc. volume	12–XII–29	Paralyzed 20–XII–29 Prostrate 22–XII–29 Killed 23–XII–29	Injected with 3½ months old concentrate without result
	71	42, 73	90–1 concentrate of filtrate	0.015 cc. in 1.5 cc. volume	12–XII–29	Remained normal	Injected with fecal concentrate from acute cases without result
3	83	42, 73	5 per cent brain cord suspension	0.15 cc. in 1.5 cc. volume	12–XII–29	Paralyzed 28–XII–29 Prostrate 29–XII–29 Dead 31–XII–29	Injected with 4½ months old concentrate without result
	84	42, 73	5 per cent brain cord suspension	0.015 cc. in 1.5 cc. volume	12–XII–29	Remained well	Injected with material from a case, not poliomyelitis
	106	67, 104	5 per cent brain cord suspension	2 cc.	29–III–30	Paralyzed 8–IV–30 Prostrate 10–IV–30 Dead 14–IV–30	Injected with small amount of filtrate in series in which none came down
	120	67, 104	5 per cent brain cord suspension	0.02 cc. in 2 cc. volume	29–III–30	Remained well	None
4	103	67, 104	40–1 concentrate of the filtrate	0.1 cc. in 2 cc. volume	29–III–30	Paralyzed 11–IV–30 Prostrate 13–IV–30 Killed 15–IV–30	Injected with 0.003 cc. suspension in series in which only 1.5 cc. undiluted suspension produced poliomyelitis
	110	67, 104	40–1 concentrate of the filtrate	0.02 cc. in 2 cc. volume	29–III–30	Paralyzed 12–IV–30 Prostrate 13–IV–30 Moribund 14–IV–30	None

TABLE 2

Viability of virus in saturated salt solution concentrates

See table 1

SERIES	MONKEY NUMBER	SOURCE OF MATERIAL	INOCULUM	AMOUNT INJECTED	DATE OF INOCULATION	SUBSEQUENT COURSE	PREVIOUS HISTORY
5	24	17	Filtrate of 5 per cent suspension	2 cc.	9–I–29	Paralyzed 14–I–29 Died 16–I–29	None
	23	17	33–1 concentrate of filtrate of 5 per cent suspension	0.06 cc. in 2 cc. volume	9–I–29	Paralyzed 17–I–29 Prostrate 18–I–29 Died 19–I–29	None
	25	17	33–1 concentrate of filtrate of 5 per cent suspension	0.03 cc. in 2 cc. volume	9–I–29	Paralyzed 15–I–29 Prostrate 18–I–29 Dead 20–I–29	None
	26	17	Same concentrate as above kept at 10° to 12°C. for 38 days	0.06 cc. in 2 cc. volume	16–II–29	Paralyzed 25–II–29 Prostrate 26–II–29 Killed 27–II–29	None
	33	17	Same concentrate kept at 10° to 12°C. for 2 months	0.06 cc. in 2 cc. volume	8–III–29	Prostrate 15–III–29 Killed	None
	43	17	Same concentration kept in refrigeration for 4 months	0.06 cc. in 2 cc. volume	10–V–29	Remained well	None

6	44	30, 33, 35	35–1 concentrate of filtrate	0.01 cc. in 1 cc. volume	21–VI–29	Paralyzed 5–VII–29 Killed 6–VII–29	Injected in a series none of which came down
	73	30, 33, 35	Same as above kept in refrigerator 4 months	0.03 cc. in 1 cc. volume	24–X–29	Prostrate 4–XI–29 Dead 5–XI–29	Injected with "final supernatant" which has never produced poliomyelitis
	74	23, 26	20–1 concentrate of filtrate refrigerated since 3–VI–29, 4½ months	0.075 in 1 cc. volume	24–X–29	Prostrate 4–XI–29 Dead 7–XI–29	Injected with precipitate from ⅓ saturation of filtrate with ammonium sulphate, series 28–VII–29. Developed tremor and excitability but recovered
	83	23, 26	20–1 concentrate of filtrate refrigerated since 3–VI–29, 4½ months	0.075 cc. in 1 cc. volume	24–X–29	Remained well	None

altered in potency. Whether this has been due to the various types of handling to which the virus has been subjected or to one of the spontaneous changes in virulence described by Flexner, Clark and Amoss (1914) is not clear. During this period, doses as large as 2 cc. of the filtrate have sometimes failed to produce the disease and when effective doses have been given, the progress of the disease has been slow, and recovery would have been successful in many of the animals had they not been killed with chloroform. Also, the individual susceptibility of an animal relatively more resistant than human beings, becomes more apparent when one is dealing with doses approximating the minimal effective amounts. One is reminded of the markedly irregular results obtained by the injection of the globoid bodies (Flexner, Noguchi and Amoss, 1915) and of the occasional successful infection following the injection of very small amounts of the filtrate.

VIABILITY OF CONCENTRATED VIRUS IN SATURATED SALT SOLUTIONS

Since the saturated salt solution resulting from the evaporation of the water did not appear to injure the virus immediately, it seemed advisable to determine the viability of the virus under these conditions so generally destructive for living cells. With the "concentrate" maintained at refrigerator temperature (10° to 12°), the virus remained fully potent for at least two months, becoming somewhat less active but still highly infective at the end of four or five months (see table 2, series 5 and 6).

Streptococci isolated by Dr. E. C. Rosenow from cases of poliomyelitis were treated in the same way as the virus, save that normal rabbit brain and cord were used to make the suspension and to the 300 cc. of filtrate were added three blood agar slants of the organisms suspended in 1.5 cc. salt solution. The whole was concentrated 30 to 1 and cultures made at intervals. Growth was obtained, following the transplantation of liberal quantities through the fifteenth day but not on the thirty-fifth day.

It is possible that the remarkable resistance of the poliomyelitis virus to the lytic effect of the saturated saline solution may be due

to the protective action of the associated proteins as shown by Olitsky and Boez (1927) for the virus of the mosaic disease of tobacco. Yet in the same medium, streptococci are destroyed much more promptly. They become shrunken and granular within three weeks.

When considered in comparison with the prolonged viability of the virus under similar conditions, this would tend to indicate the absence of causal relation between the streptococci and poliomyelitis.

ATTEMPTED CULTIVATION AND MICROSCOPIC EXAMINATION

Many attempts have been made to cultivate organisms from the concentrates, and later from the dialyzed concentrates, and to study smears stained by a variety of methods. The media employed were glucose brain broth, Smith-Noguchi medium, blood agar, Locke's egg medium, ascitic fluid broth and agar and ordinary beef infusion agar and broth. All told, about twenty concentrates from thirty monkeys have been planted and subsequently examined for growth. Occasionally contaminations have been found, staphylococci, a diphtheroid, aerobic spore formers and finally, in one instance only, a streptococcus. This streptococcus was of the viridans type and fermented lactose, mannitol and salicin. It produced no apparent symptoms in a monkey when one-half of a blood agar culture was injected directly into the brain substance.

Direct microscopic examination of the concentrates, using a variety of staining technics, has shown nothing that has not been found in normal brain cord suspensions treated in the same way. If streptococci were etiologically related to poliomyelitis, we should expect to find them in smears and cultures from these highly concentrated filtrates.

PRECIPITATION OF THE VIRUS WITH VARIOUS PROTEIN FRACTIONS

a. Water soluble and water insoluble fractions

The presence of the large amount of salt in the concentrate proved, however, to cause difficulty in the examination of the

TABLE 3

Separation of the virus with various protein fractions

See table 1

SERIES	ANIMAL NUMBER	SOURCE OF MATERIAL	INOCULUM	AMOUNT INJECTED	DATE OF INOCULATION	SUBSEQUENT COURSE	PREVIOUS HISTORY
			a. Separation by dialysis				
7	38	15	Precipitate from dialysate of concentrate (euglobulin)	2 cc. representing 100 cc. of original filtrate	23–III–29	Remained well	None
	35	15	Supernatant or water soluble protein fractions (pseudoglobulins and albumens) from dialysate of concentrate	1 cc. representing 12 cc. of original filtrate	19–III–29	Paralyzed 27–III–29 Prostrate 28–III–29 Died 29–III–29	None
8	49	30, 33, 35	Precipitate from dialysate of concentrate	1 cc. representing 45 cc. of original filtrate	21–VI–29	Remained well	Injected with albumen fraction from dialysate in series of 17–V–29
	46	30, 33, 35	Supernatant after dialysis of concentrate	1 cc. representing 1.5 cc. of original filtrate	21–VI–29	Tremor, excitable 5–VII–29 Tremor and ataxia 6–VII–29 Recovered; subsequently survived intracerebral inoculation with potent suspension	Injected with 0.01 cc. suspension of 37, in series 16–V–29. None down

	66	39, 60	Precipitate from dialysate of concentrate	2 cc. representing 140 cc. of original filtrate	31–VII–29	Remained well	None
9	43	39, 60	30–1 concentrate of filtrate	0.05 cc. in 1.5 cc. volume	31–VII–29	Paralyzed 9–VIII–29 Prostrate 10–VIII–29 Killed 16–VIII–29	Injected with old concentrate. See table 2, series 5. Subsequently shown to withstand at least 3 fatal doses in series 21–VI–29
	32	25	Refiltered dialysate of concentrate	0.2 cc. in 2 cc. volume representing 2 cc. original filtrate	4–IV–29	Prostrate 19–IV–29 Dead 22–IV–29	Injected with small dose of virus without effect
10	42	25	Refiltered dialysate of concentrate	0.05 cc. in 0.5 cc. volume representing about 0.5 cc. original filtrate	4–IV–29	Paralyzed 17–IV–29 Prostrate 18–IV–29 Moribund 19–IV–29	None
11	62	30, 33, 35	Diffusate (after dialysis of concentrate) concentrated and then dialyzed with resulting concentration of 25–1	2 cc. icer., 10 cc. iper.	22–VI–29	Remained well	None

TABLE 3—*Continued*

SERIES	ANIMAL NUMBER	SOURCE OF MATERIAL	INOCULUM	AMOUNT INJECTED	DATE OF INOCULATION	SUBSEQUENT COURSE	PREVIOUS HISTORY
b. Separation by half saturation with ammonium sulphate							
12	59	30, 33, 35	Globulin fraction from dialysate (16–1)	2 cc. icer., 6 cc. iper. representing about 40 cc. of filtrate per cubic centimeter	22–VI–29	Paralyzed 30–VI–29 Prostrate 1–VII–29 Killed 3–VII–29	None
	66	61, 62	Globulin fraction from dialysate (16–1)	2 cc. icer., 1 cc. iper. representing about 70 cc. of filtrate per cubic centimeter	27–VIII–29	Paralyzed 6–IX–29 Gradual recovery with residual paralysis	Injected the water insoluble proteins from dialysate without effect. Series 31–VII–29
	60	30, 33, 35	Globulin fraction from filtrate	2 cc. icer., 6 cc. iper. representing about 15 cc. of filtrate per cubic centimeter	22–VI–29	Paralyzed 28–VI–29 Prostrate 29–VI–29 Died 2–VII–29	None
	74	61, 62	Globulin fraction from filtrate	0.75 cc. representing about 50 cc. of filtrate per cubic centimeter	28–VIII–29	Tremor, excitability and ataxia 6–IX–29 Recovered; subsequently succumbed to poliomyelitis, following injection of concentrate 24–X–29	None

c. Separation following complete saturation with ammonium sulphate

	MONKEY NUMBER						
13 and 14	51	30, 33, 35	Albumen fraction from dialysate	2 cc. icer., 6 cc. iper.; each cubic centimeter represents 70 cc. filtrate	22–VI–29	Remained well	Injected with concentrated diffusate without effect
	67	39, 60	Albumen fraction from dialysate	2 cc. icer., 8 cc. iper.; each cubic centimeter represents 10 cc. original filtrate	31–VII–29	Remained well	None
	72	61, 62	Albumen fraction of dialysate *but* globulins incompletely removed by centrifugation	2 cc. icer., 2 cc. iper.; each cubic centimeter represents 60 cc. filtrate	27–VIII–29	Paralyzed and prostrate 6–IX–29 Dead 9–IX–29	None
	61	30, 33, 35	Albumen fraction of filtrate	2 cc. icer., 6 cc. iper.; each cubic centimeter represents 20 cc. filtrate	22–VI–29	Paralyzed and prostrate 29–VI–29 Killed 29–VI–29	None
	62	39, 60	Albumen fraction of filtrate	2 cc. icer., 8 cc. iper.; each cubic centimeter represents 3 cc. filtrate	31–VII–29	Paralyzed and prostrate 13–VIII–29 Killed 15–VIII–29	Injected with concentrated diffusate without effect

TABLE 3—*Concluded*

SERIES	MONKEY NUMBER	SOURCE OF MATERIAL	INOCULUM	AMOUNT INJECTED	DATE OF INOCULATION	SUBSEQUENT COURSE	PREVIOUS HISTORY
c. Separation following complete saturation with ammonium sulphate—*Concluded*							
13 and 14	75	61, 62	Albumen fraction of filtrate obtained following *prolonged* centrifugation after half saturation	2 cc.; each cubic centimeter represents 30 cc. filtrate	28–VIII–29	No poliomyelitis	None
d. Final supernatant after complete saturation							
15	68	39, 60	Dialyzed and concentrated final supernatant after complete saturation with ammonium sulphate	2 cc. icer., 8 cc. iper.; each cubic centimeter represents 3 cc. of final supernatant	31–VII–29	Remained well	None
	73	61, 62	Dialyzed and concentrated final supernatant after complete saturation with ammonium sulphate	2 cc. icer., 8 cc. iper.; each cubic centimeter represents 2 cc. of final supernatant	27–VIII–29	Remained well	None

smears and was obviously also a handicap to its use in animals unless redilution were practiced.

Hence, the concentrate was dialyzed with aseptic precautions against sterile distilled water in autoclaved bags made with celloidin dissolved in equal part of alcohol and ether. Later in the work, cellulose sausage casings made by the Visking Corportion of Chicago were employed. The dialysis was continued until the diffusate no longer gave a test for chlorides upon the addition of silver nitrate solution. With the loss of salt, a rather heavy brownish-green precipitate was thrown out of solution and easily removed by centrifugation leaving a clear, straw-colored supernatant.

The precipitates, secured in this manner from three different concentrates made from filtrates from six animals, have been tested for virus. After three washings in distilled water, the sediment has been taken up in a small volume of physiologic saline in which it is largely redissolved and amounts equivalent to as much as 140 cc. of the original filtrate have then been injected intracerebrally into monkeys. In every instance the animals have remained entirely well (see table 3, series 7, 8 and 9). The clear supernatant on the other hand has in every case proved effective in producing the disease, although in one instance (monkey 46) the animal did not become definitely paralyzed.

A preliminary but not precise titration of the dialysate indicates that there is little loss of potency during the process, due allowance being made for the increase in volume (table 3, series 10).

The diffusate, on the other hand, after the complete dialysis of the concentrate, has in one instance been concentrated and subsequently dialyzed free from sodium chloride. This material was injected into monkeys without producing poliomyelitis (table 3, series 11).

It would seem reasonable to conclude therefore that the virus is not diffusible through a celloidin membrane, is not present in effective amounts in the water-insoluble proteins even when the precipitate from a volume of concentrate equivalent to 140 cc. of the original filtrate has been injected, and that it does remain constantly associated with the water soluble proteins.

Further evidence along this line was sought by precipitating both the dialyzed concentrate and the regular 5 per cent filtrate with ammonium sulphate.

b. Fraction precipitated by half saturation with ammonium sulphate

After the water insoluble fraction had been removed from the dialysate by centrifugation, a grayish precipitate was secured by adding an equal volume of sterile saturated ammonium sulphate solution. This precipitate was removed by centrifugation and washed three times with half saturated ammonium sulphate solution. This washed sediment was then taken up in a small volume of distilled water and dialyzed against sterile distilled water until the diffusate showed no trace of sulphates upon the addition of barium chloride solution and the dialysate no odor of ammonia upon the addition of strong sodium hydroxide to a test portion.

A similar procedure was carried out with the virus filtrate.

In both instances, the animals injected with the dialyzed precipitate obtained from the dialysate came down typically while one of the animals (60) injected with the similar material obtained from the filtrate came down typically and the other (74) developed a definite tremor, excitability and ataxia on the ninth day but the disease did not progress. Seven weeks later this animal developed poliomyelitis following the injection of concentrate. In each of these instances the equivalent of relatively large amounts of virus was injected and no attempt has as yet been made to obtain quantitative information (table 3, series 12).

c. The fraction precipitated by complete saturation with ammonium sulphate

To each 100 cc. of the supernatant secured after half saturation as described above, were added 35 grams of sterile ammonium sulphate. After shaking the mixture and allowing it to stand for fifteen to twenty minutes, a light flocculent precipitate appeared which could be removed only by prolonged centrifugation (thirty to sixty minutes). This precipitate was then washed three times

with saturated ammonium sulphate and was resuspended in sterile distilled water and dialyzed until the diffusate showed no trace of sulphates.

The results after inoculating this material were at first confusing. In two series, the animals (51 and 67) that received this albumen fraction from the *dialysate*, did not develop poliomyelitis while in the same two series, the animals that received the similar fractions from the *filtrate* developed typical poliomyelitis. The most plausible explanation of the difficulty seemed to be the marked difference in the volumes employed and the resulting difference in the effect of centrifugation. Two hundred cubic centimeters of filtrate had been employed whereas only 25 to 50 cc. of the dialysates had been employed. This fact made it possible to remove the precipitate resulting from half saturation with ammonium sulphate more completely in the case of the dialysate than in the case of the filtrate. Accordingly, in the next series, prolonged centrifugation was used following the half saturation of the filtrate before completely saturating the supernatant with ammonium sulphate. The precipitate so secured did not produce poliomyelitis whereas the half saturation precipitate did (monkey 75). At the same time the half saturation portion of the dialysate was less completely centrifuged than usual so that the supernatant was not completely clear. Following this procedure, the fraction obtained by complete saturation did produce poliomyelitis in a monkey (see table 3, series 13 and 14).

The final supernatant after the last precipitation by complete saturation, was also tested in two series for the presence of the virus and in neither instance did it produce any effect in animals. This supernatant was dialyzed free from ammonium sulphate and then concentrated to a few cc. and injected (see table 3, series 15).

There seems little doubt therefore that the virus is precipitated with the globulin fraction by ammonium sulphate and that as the earlier dialysis experiments indicate, the pseudo or water soluble globulins are the essential carriers of the virus.

In one instance each, the precipitates obtained by the addition to the virus of two volumes of absolute alcohol in the one case and

three volumes of acetone in the other, produced no result when injected intracerebrally into monkeys.

USE OF CONCENTRATION IN VACUO IN DETERMINING THE INFECTIVITY OF FECES AFTER FEEDING MONKEYS WITH THE VIRUS

The demonstration by Aycock and others that poliomyelitis may apparently be spread through milk supply, raises the question whether the milk is contaminated by droplets from the nasopharynx or whether the virus may occasionally pass through the feces unaltered and thus reach the milk supply. Also, the possibility that the infection may in these milk borne epidemics actually take place through the stomach or the intestines rather than the nasopharynx is again suggested.

Accordingly, 3 monkeys were fed about once in five days by stomach tube with 20 to 25 cc. brain cord suspensions, two for a period of fourteen weeks with a total of 310 cc. each and one for eight weeks with a total of 215 cc. These animals did not develop poliomyelitis and subsequently all came down typically following intracerebral injection of the virus. This is in accord with earlier observations by Flexner and Lewis (1910), and with a more recent report by Schultz, (1929). Leiner and v. Wiesner, through the use of sodium bicarbonate to neutralize the hydrochloric acid of the stomach and large doses of morphia to reduce the peristalsis, succeeded in producing the disease in 2 out of 5 monkeys fed, but Amoss (1928) following their procedure and giving even larger doses did not confirm their observations.

During the course of this feeding experiment, after the animals had been fed a total of 150 cc. each over a period of approximately a month, they were, after two successive feedings of 20 cc. each, placed at once in clean, sterile cages. The feces of the three monkeys were collected each time over a period of forty-eight hours, resulting in a total of 65 grams. A 10 per cent suspension was made, shaken, centrifuged and the supernatant filtered through sterile Mandler filters. The 500 cc. of filtrate obtained were concentrated *in vacuo* to 10 cc., and dialyzed free of sodium chloride, thus being increased in volume to 30 cc. One and one-fourth cubic centimeter of this dialysate, representing roughly the salt

solution soluble equivalent of the feces passed by one monkey in about twelve hours, was injected intracerebrally into monkey 39. Following an incubation period of six days, the monkey developed typical symptoms of poliomyelitis which became progressively worse, with complete paralysis and a fatal termination two days later. The identity of the disease was confirmed by the microscopic findings and by reinoculation into another monkey with the production of typical poliomyelitis in that animal also (see table 4, series 16).

TABLE 4

Infectivity of feces after feeding monkeys with the virus

See table 1

SERIES	MONKEY NUMBER	SOURCE OF MATERIAL	INOCULUM	AMOUNT INJECTED	DATE OF INOCULATION	SUBSEQUENT COURSE	PREVIOUS HISTORY
16	39	A variety of suspensions fed to monkeys 19, 21, 30	Dialyzed concentrate of feces from monkeys fed with virus	1.25 cc. representing feces passed in about 12 hours	23–III–29	Paralyzed 30–III–29 Dead 31–III–29	None
	54	39	Suspension of brain cord	1.5 cc.	31–V–29	Paralyzed 7–VI–29 Prostrate 8–VI–29 Killed 11–VI–29	None

In five series of experiments with a total of ten monkeys injected, we have tried to repeat this experiment without, however, using the month's preliminary feeding which seemed to us not involved in the immediate question. The duration of time of feeding of the virus prior to the beginning of collection has varied from thirty minutes to four days; the duration of time of collection after feeding has varied from sixteen to forty-eight hours; the virus has also been fed during the period of collection in amounts from 20 to 150 cc. in twenty-four hours; suspensions from glycerinated and from fresh tissues have been employed; and

the dialysates have been injected not only intracerebrally in volumes of 1 to 3 cc. but intraperitoneally in volumes of 2 to 10 cc. All of these have given negative results; so we are forced to conclude that the virus does not pass through the intestinal tract of monkeys even when fed in enormous amounts save under exceptional and unknown conditions. Also, feces collected from 3 groups of monkeys (8 in all) during the acute stages of the disease were collected and carried through the same procedure. The three monkeys inoculated with these dialyzed concentrates, remained well. By exclusion, the importance of the upper respiratory tract in the transmission of poliomyelitis is again emphasized.

SUMMARY AND CONCLUSIONS

It has been possible to concentrate poliomyelitis virus by distillation *in vacuo* so that the residue is more potent than the original filtrate or the brain cord suspension from which the filtrates are made.

The virus remains potent in the "concentrate," even though the solution contains sodium chloride to saturation, for more than four months.

On the other hand, streptococci, isolated from cases of poliomyelitis, concentrated in a filtrate of normal brain cord suspension, are no longer viable after thirty-five days. This would tend to discredit the streptococcus as the specific etiologic agent in poliomyelitis.

The "concentrates" can be dialyzed against sterile distilled water without essential loss of potency.

The virus is present in the water-soluble portion of these dialysates and not in the water-insoluble or euglobulin fraction.

The virus is not demonstrable in the concentrated diffusate.

The virus is present in the globulin precipitate, obtained either from the regular filtrates or from the "dialysates," following half saturation with ammonium sulphate.

Complete saturation of the thoroughly centrifuged supernatant so obtained, gives a precipitate (albumen fraction) which does not contain the virus.

The virus is not demonstrable in the concentrated final supernatant after complete saturation with ammonium sulphate.

These fractional precipitations together with the facts obtained following dialysis indicate that the virus remains constantly associated with the pseudoglobulin fraction of the proteins.

In one out of six experiments, the feces, obtained following the feeding of large volumes of poliomyelitis virus, gave an inoculum with which the disease was experimentally produced. The concentration of feces from acute cases gave negative results in the three groups investigated. These experiments would indicate that the virus does not commonly pass through the intestinal tract of monkeys in an infective form.

Many attempts have been made to cultivate a significant organism from the highly infective concentrates and dialysates with negative results. Streptococci were found as a contaminant in one instance only. Stained smears have also shown nothing that could not be found in the "dialysate" from normal sources.

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CARBOHYDRATE METABOLISM OF ORGANISMS OF THE GENUS MYCOBACTERIUM[1]

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I. INTRODUCTION

The present article deals with various aspects of carbohydrate utilization by the organisms of the genus Mycobacterium when meat infusion broth constitutes the basis of the media in which the organisms are grown. A correlated study in which a synthetic media was used will be reported in a subsequent article.

There is a scarcity of reported research dealing directly with the problem of carbohydrate utilization by organisms of the genus Mycobacterium. Most of the reported work, wherein plain broth has been used as the basis of the media, provides no satisfactory quantitative information as to the extent of carbohydrate utilization. The reason for this will become apparent from subsequent considerations.

Until quite recently, direct quantitative estimations have not been extensively applied to the determination of carbohydrate utilization by bacteria. In the case of the vast majority of bacteria that have been studied, there is associated with the utilization of carbohydrates the production of various organic acids as cleavage products. The accumulation of these acids in the media results in an increase in hydrogen ion concentration. By the use of appropriate indicators this acid production has been readily

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demonstrable. This has constituted the most commonly used criterion for carbohydrate utilization.

There has been encountered no increase in acidity accompanying the growth of organisms of the genus Mycobacterium when grown in carbohydrate broth (Merrill, 1928, 1929). Using pH changes as an index it is then not clear whether any of the organisms of this group do, or do not, utilize any carbohydrates.

Theobald Smith (1905) long since demonstrated clearly that the addition of glycerol to broth increased the growth of *Myco. tuberculosis* (*hominis*). He also demonstrated a terminal acidity when this organism was grown in 3 per cent glycerol broth. The first reaction change, however, was distinctly to the alkaline side. The usual criterion of early acid production thus does not hold even with glycerol. That the presence of glycerol increases the growth of the human type of *Myco. tuberculosis*, however, cannot be doubted. This suggests that glycerol is utilized. In the case of *Myco. tuberculosis* (*bovis*) the reaction was shown by Smith to become progressively alkaline in glycerol broth. The evidence of utilization is thus inconclusive.

Kendall, Day, and Walker (1914) in working with several species of this genus, including some saprophytic strains, did not in a single instance obtain a primary acidity. They stated that evidently there was an acid production from the glycerol but that it was a "differential acidity," alkaline products being formed in excess. They were, nevertheless, convinced that the glycerol was utilized, but were of the opinion that glucose and mannitol were not utilized. Similar results were later reported by Long and Major (1922). Gamble and Herrick (1922) report utilization of glucose by the tubercle bacillus.

Work done by Frouin and Guillaume (1923) would indicate that some carbohydrates are utilized by *Myco. tuberculosis* (*hominis*) and their later work (1926) leaves little doubt that some of the carbohydrates are utilized.

The work of Braun et al. (1924, 1924a, 1924b, 1925), which will be considered more fully in a subsequent report, gives quite conclusive evidence that carbohydrates and their derivatives can be utilized by many of the organisms belonging to the genus Myco-

bacterium, at least when these organisms are grown in synthetic media.

There are still some workers, however, who believe that aside from glycerol there is no utilization of carbohydrates or their derivatives by these organisms. Recently Weinzirl and Knapton (1927) from their results of an extensive study of the reaction curves of fifteen species of bacteria of the genus Mycobacterium on synthetic media stated that glucose, mannitol, and lactose are apparently not utilized. Their methods, however, were not designed to determine whether the carbohydrates were utilized since reaction curves constituted their only criterion for utilization. As will be shown below, results based upon such methods are indefinite.

A problem quite fundamental in character thus presents itself. The Mycobacteria apparently utilize carbohydrates without the production of acids. How, then, does the carbohydrate metabolism of these organisms differ from that of most bacteria? What is the extent of carbohydrate utilization by the Mycobacteria? What are the end products? How does the presence of carbohydrates in a medium affect protein metabolism?

II. ORGANISMS USED IN THIS STUDY

The organisms studied comprise a number of the so-called "acid-fast" microörganisms classified in the genus Mycobacterium. In all, nineteen organisms were used. The species names and sources from which these were obtained were as follows:

1. *Mycobacterium tuberculosis* (*hominis*). Three strains of this organism were used:

a. H-37 strain obtained from the American Type Culture Collection of Chicago.

b. M-1 strain which was isolated by the author from the sputum of a tuberculosis patient at the Veterans Bureau Hospital located at Jefferson Barracks, Mo. This strain was definitely pathogenic for guinea pigs.

c. A saprophytic strain which has been carried in stock in the laboratory for some years. This stain was originally isolated from human sputum. It grows rapidly and is now non-pathogenic for guinea pigs.

2. *Mycobacterium tuberculosis* (*bovis*) No. 112 of the American Type Culture Collection of Chicago.

3. *Mycobacterium leprae.* Six strains of this organism were used. All were obtained from the American Type Culture Collection of Chicago. Their serial numbers are as follows: 282, 65, 66, 67, 68 and 69.

4. *Mycobacterium avium,* No. 118 of American Type Culture Collection.

5. *Mycobacterium ranae,* No. 110 of American Type Culture Collection.

6. *Mycobacterium chelonei,* No. 114 of American Type Culture Collection.

7. *Mycobacterium smegmatis,* a laboratory culture the exact origin of which is unknown.

8. *Mycobacterium butyricum,* No. 357 of American Type Culture Collection.

9. *Mycobacterium berolinensis,* No. 356 of American Type Culture Collection.

10. *Mycobacterium friburgensis,* obtained some years ago from the University of Pennsylvania.

11. *Mycobacterium stercusis,* No. 77 of American Type Culture Collection.

12. *Mycobacterium phlei,* a laboratory culture, the exact origin of which is unknown.

13. *Mycobacterium piscium,* No. 111 of American Type Culture Collection.

III. REACTION CHANGE DETERMINATIONS

The first undertaking was to determine if there occurred a change in reaction towards the acid side as a result of growth of each of these organisms in glucose broth. In every instance it was observed that the reaction of the media became progressively more alkaline. This was found to be the case in carbohydrate broth as well as in plain broth, i.e., there was no evidence of accumulation of acid in the media. These results are in agreement with those of previous workers (Smith, Kendall et al.). This eliminated any possibility of direct determination of carbohydrate utilization by the generally used indicator method.

Kendall et al. (1914) and Long (1922) determined the reaction

curves of several organisms of this group and observed that these curves differed with the different carbohydrates employed. They were not concerned with the demonstration of the utilization of carbohydrates but it seemed possible that in this study variations in the reaction curves induced by the addition of various carbohydrates might be employed as a method of determining whether the respective carbohydrates were or were not utilized.

To this end the following experiments were carried out. Various factors which might be concerned in reaction curve determinations are first considered; typical reaction changes as observed with various carbohydrates are then presented.

Methods

Standard meat infusion broth, to which 200 mgm. secondary potassium phosphate per liter was added, was used throughout as the basic medium for the reaction curve determinations. The method of determining the reaction changes in the cultures was essentially the same as that used by Long (1922); phenol sulphonphthalein (phenol red) in a concentration of 0.001 per cent being used as the indicator, Clark (1920) buffer systems being used for the standards, and the Dernby and Avery (1918) comparator block method being used in making comparison. The standards gave a definite color change from pH 6.8 to pH 8.0 and approximations of reasonable accuracy could be made down to pH 6.0 and up to pH 8.6, thus giving a satisfactory range for pH determinations.

Tubes of uniform size (18 by 150 mm.) were used throughout the entire work. In the preliminary experiments the media were adjusted to pH 7.2 to 7.4, sufficient carbohydrate was added to make a 1 per cent solution, the media tubed accurately with 10 cc. per tube, and sterilized at 15 pounds for fifteen minutes. One sterilization was adopted, since this yields less hydrolysis (Mudge, 1917).

Most of the organisms of this group are especially adapted for this type of study due to their obligate aerobic requirement which causes them to grow in the form of a pellicle at the surface, leaving the underlying liquid clear. In inoculating the tubes due recog-

nition was given this fact. The inoculum was made to float if possible and, where not possible, it was smeared on the sides of the tube at the surface of the media, care being taken in every case to make certain that some of the inoculum was in contact with the surface of the liquid medium. This insured an early and more or less uniform growth. In some of the later determinations a thin slice of cork was placed in each tube, prior to sterilization, and the inoculum placed on the cork, making it easier to keep the growth floating throughout the duration of incubation.

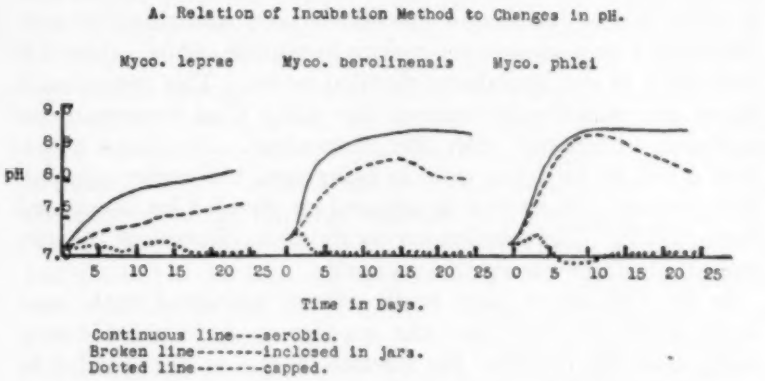
Some of the crude preliminary tests are considered worthy of mention because of the general principles concerning the metabolism of this group of organisms which are therein illustrated, and also because they throw new light on previous work in which reaction changes in the cultures were studied, especially that of Long and Major (1922).

The variations in reaction changes in the media induced by varying the method of incubation of the cultures were determined. Three organisms, *Myco. leprae*, *Myco. berolinensis*, and *Myco. phlei* were each inoculated into three tubes of plain broth. The three parallel cultures of each organism were incubated under the following conditions: Series I was incubated freely exposed to the relatively dry air of the incubator. In Series II the tubes were incubated in a closed jar, the bottom of which was covered with water. In series III the tubes were capped with celloidin caps. The reaction curves shown in figure 1, *A*, illustrate the pH changes observed.

A distinct growth with the formation of a pellicle took place in every tube, but the growth was much less in the capped tubes (series III) than in either of the other two, the most luxuriant growth being obtained in the tubes incubated under aerobic conditions (series I).

To better illustrate the effect of free access of air, a capped tube of *Myco. smegmatis* which had been incubated for nine days with little reaction change was opened to the air by puncturing a small hole in the cap. In the next five days the reaction changed from pH 7.4 to pH 8.6 and a heavy pellicle replaced the frail pellicle which had been present at the time of the puncture. These

results indicate that when the tubes are capped there is, in addition to an inhibition of growth, an accumulation of some substance which prevents a change in the reaction of the media towards the



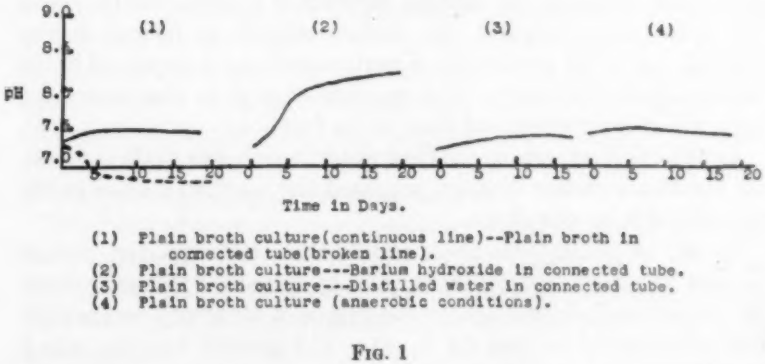

B. Relation of Carbon Dioxide and Oxygen Content of Culture Tubes to Changes in pH.
(Myco. berolinensis)
(1)
(2)
(3)
(4)
pH
9.0
8.5
8.0
7.5
7.0
0 5 10 15 20 0 5 10 15 20 0 5 10 15 0 5 10 15 20
Time in Days.
(1) Plain broth culture(continuous line)--Plain broth in connected tube(broken line).
(2) Plain broth culture---Barium hydroxide in connected tube.
(3) Plain broth culture---Distilled water in connected tube.
(4) Plain broth culture (anaerobic conditions).

FIG. 1

alkaline side. That this substance is carbon dioxide and the effect which it exerts upon the reaction changes was shown by the following experiment:

Three tubes of plain broth to which indicator (phenol red) had been added, were inoculated with each organism. One of these inoculated tubes was connected by means of a "U" shaped glass tube bearing a rubber stopper at each end to an uninoculated tube of plain broth. A second inoculated tube was similarly connected to a tube containing barium hydroxide, while a third was connected to one containing distilled water. This permitted a direct gas interchange between the tubes thus connected but excluded interchange with the atmosphere. To insure a seal that would be air tight, prior to being used the rubber stoppers were cleaned and soaked in glycerol as directed by Novy and Soule (1925). The reaction curves shown in figure 1, *B* (*1*), (*2*), and (*3*) illustrate the results obtained.

In the first set, a plain broth culture connected with plain broth, it will be noted that the reaction in the inoculated tube changed to pH 7.6 while the reaction in the uninoculated tube changed to pH 6.8. This would indicate that carbon dioxide is produced by the culture, but that decrease in pH is prevented by simultaneous production of akaline products, since there is a decrease in the pH in the connected tube which is subjected to the same carbon dioxide tension.

In the second set, a plain broth culture connected with barium hydroxide solution, the barium hydroxide solution contained in the side tube absorbed the carbon dioxide as fast as it was formed. A white precipitate of barium carbonate appeared in the barium hydroxide tube. The reaction change in the inoculated tube was more pronounced than in the first set.

In the third set, where distilled water, which has little capacity for absorbing carbon dioxide, was used the reaction change in the inoculated tube was slight.

It will be noted from these results that, although the carbon dioxide was taken out by the barium hydroxide as fast as formed, the culture still did not obtain the degree of alkalinity reached by free exposure to air (see fig. 1, *A*). The growth was also much less abundant. As other tests which were run showed that the reaction change was still less under more anaerobic conditions, it seemed reasonable to assume that the factor concerned in the

lesser amount of growth in this set was the inadequacy of oxygen required for growth.

When pyrogallic acid and sodium hydroxide were used to absorb the oxygen from the culture tubes, applying the reagents to the cotton stopper and sealing the tube with rubber stoppers and paraffine (technique of Rockwell, 1924) there was obtained no appreciable change in reaction and a very scanty growth of the saprophytic forms, *Myco. phlei* and *Myco. berolinensis*, after twenty days of incubation. These reaction changes are illustrated in figure 1, *B* (*4*). From a comparison of these results with those obtained when the barium hydroxide was present in a side tube (fig. 1, *B* (*2*)) it is evident that the pH change was distinctly less marked in the tube deprived of oxygen. The carbon dioxide was absorbed by the alkali in both cases, the difference being purely one of oxygen present, a diminution of which caused a corresponding diminution of growth. Evidently, then, it is a general characteristic throughout this group of organisms that, other factors being favorable, the growth is proportional to the oxygen supply. This was conclusively shown to be the case with the tubercle bacillus by the work of Novy and Soule (1925). Any method involving a study of the reaction changes of media which fails to take this factor into consideration would thus of necessity be faulty. Certainly in the light of this consideration the method of determining the reaction curves in tubes capped with tin foil as used by Long and Major (1922) is at best approximate and open to error. Any method of sealing that does not permit of a free gas interchange with the atmosphere, or a constant oxygen and carbon dioxide tension, would be certain to introduce variation in the reaction changes from tube to tube.

The facts presented above suggested the necessity of refraining from interfering with free oxygen supply during the incubation, and for that reason the incubator atmosphere was maintained at a high relative humidity and the tubes were stoppered only with cotton plugs.

In a preliminary series of determinations plain broth, glucose, lactose, sucrose, mannitol, and dextrin broths were used. By the method of sterilization mentioned above it was impossible to

obtain media of the same constant pH. Although all the broth to which the various carbohydrates were added was taken from the same container the pH of the media following sterilization were respectively: plain broth, 7.4; sucrose broth 7.3; dextrin broth, 7.1; glucose broth, 7.0; lactose broth, 7.1; and mannitol broth, 7.3. This eliminated the possibility of making an absolute comparison but the tests were run through to determine whether the difference in the reaction curves would be pronounced enough to indicate a utilization of any of the carbohydrates. With few exceptions, the results were too indefinite to be of any value. Invariably, the change of reaction was towards the alkaline side and the terminal reactions in different media by a given organism were practically the same.

An attempt was made to substitute sterilization by filtration subsequent to the addition of the carbohydrates to the media. This insured an identity of the various media, save for the presence of the different carbohydrates, at the beginning of the determinations. To the broth, which had previously been sterilized in the autoclave, the indicator was added and the reaction of the broth was adjusted to pH 7.2. The carbohydrates were weighed out and added in a 1 per cent concentration. The medium was filtered immediately, using Grade "N" Berkefeld filters. It was then tubed, approximately ten cubic centimeters per tube. Subsequent to filling, the tubes were incubated for at least three days as a check on their sterility.

In the tests reported plain broth, 1 per cent mannitol broth, 1 per cent galactose broth, and 1 per cent glucose broth were used. Each was run in duplicate and the mean pH of the two tubes in the few cases in which they differed was recorded in plotting the reaction curves. In the series reported in figure 2 the incubation was for a duration of seventeen days. It should be remembered in reading the results from the graphs that beyond pH 8.2 the determinations are more or less approximate.

There is no observable change in the color of the indicator used beyond pH 8.6. Consequently none of the curves indicate an alkalinity exceeding this figure, but in the light of later work there is little doubt that in many cases the media became somewhat more alkaline than shown in the graph.

Results

The reaction curves obtained from the plain broth control cultures (first column of fig. 2) present several interesting aspects. The most striking feature is the general similarity of the curves. A close metabolic relationship between the various members of the group is thereby indicated. The typical curves, such as that of *Myco. tuberculosis* (*hominis*), present a short period of lag (or absence of reaction change) followed by a rapid increase in alkalinity with subsequent flattening out of the curve. During the lag period a pellicle was formed on the surface of the media and the rapidity of the rise in the pH may be roughly stated to be proportional to the rapidity of growth.

There are, among the curves from the plain broth cultures (first column), a few that vary more or less from the so-called typical curves. In the culture of *Myco. leprae* development of alkalinity is shown to have been somewhat less rapid. This was associated with a less rapid growth but, as will be pointed out later, this is not the only factor concerned in the case of this particular organism, since later quantitative tests have demonstrated that *Myco. leprae* produces less ammonia than the other organisms of the group which were studied.

Again, the period of lag was longer in the culture of *Myco. chelonei.* The optimum temperature for the growth of this organism is about 20°C. but growth even at this temperature is less rapid than that observed for the other organisms at their respective optimal temperatures. The lessened rapidity of growth may thus account for the different type of reaction change. The slight dipping of the curve after fifteen days may be considered to be due to difficulties in the determination of the pH, since an error of pH 0.2 may readily be made beyond pH 8.2 with the indicator used. No such tendency to late diminished alkalinity has been noticed in other tests employing this organism.

The variation of the curve for *Myco. phlei* from the typical is well within the error of the experimental method.

The curves shown in the second column, which represent the reaction changes in mannitol broth cultures of the respective

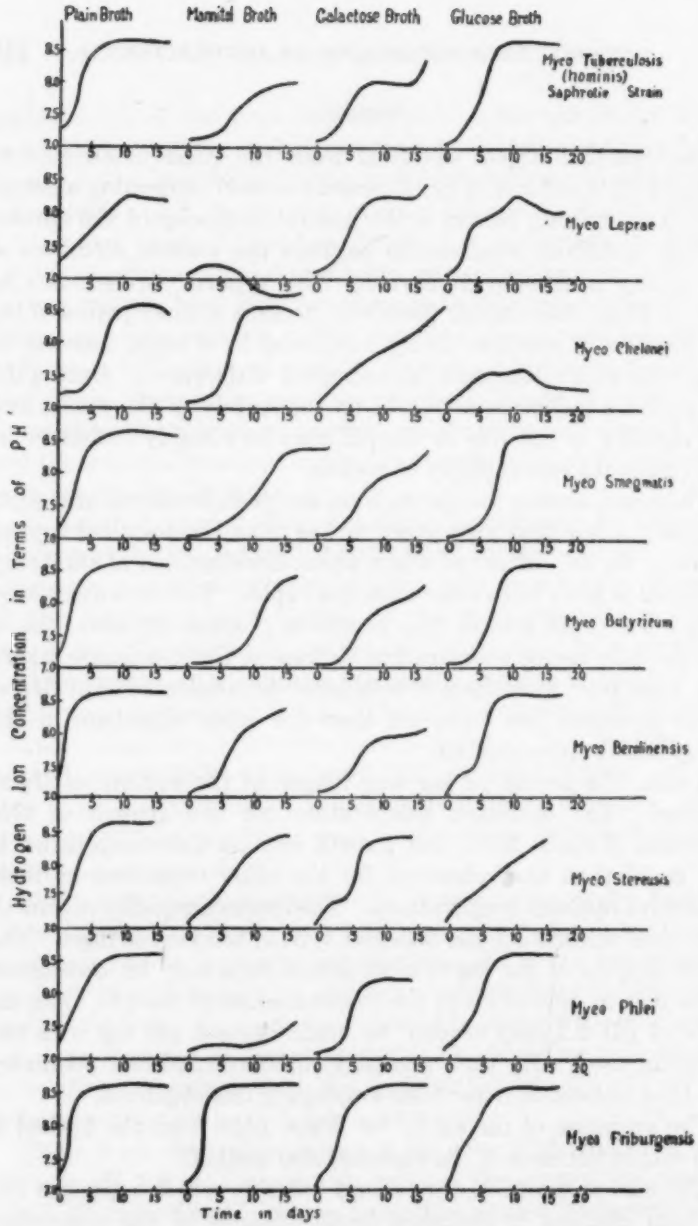


FIG. 2. REACTION CHANGES INDUCED IN MEDIA BY ORGANISMS OF THE GENUS MYCOBACTERIUM

organisms, show more variation. In general, it will be noted that the reaction here also changes towards progressive increased alkalinity, but with less rapidity than in the plain broth controls. This characteristic is likewise observable in the third and fourth columns (galactose and glucose broth cultures). As will be shown below, this tendency is apparently due to the protein-sparing action exerted by the carbohydrates, as a result of which less alkaline protein cleavage products, especially ammonia, accumulate in the media. It must be emphasized, however, that two opposing variable factors are involved. The above factor tends to inhibit protein cleavage and hence reaction change of the media, while the presence of utilizable carbohydrates tends to increase the rapidity of growth of the organisms. Although diminished, there is nevertheless some protein cleavage associated with growth in carbohydrate-containing media. If the rate of growth is sufficiently increased by the presence of the carbohydrate, the protein sparing affect may be counterbalanced and the reaction change may be as rapid as in the plain broth control culture. Such would appear to be the explanation of the curves with *Myco. friburgensis* in mannitol broth, which shows no variation from the curve of the plain broth culture, since this organism has been shown to utilize mannitol (results to be reported in later paper). Utilization of mannitol by *Myco. chelonei* has not been demonstrated. This curve likewise presents no variation from that of the plain broth control.

At the other extreme, protein cleavage may be so retarded by the presence of utilizable carbohydrates that, despite a more luxuriant growth than that present in the plain broth control, there is only slight increase in alkalinity. This is exemplified by the curve for *Myco. phlei* in mannitol broth. A still greater extreme is the curve shown in the same column for *Myco. leprae.* Subsequent tests have shown that the secondary slight decrease in pH in the case of the latter organism is variable but the alkalinity has in no instances been noted to exceed pH 7.4. It is significant that in the quantitative determinations an actual decrease in the ammonia content of the media has been observed in some carbohydrate broth cultures of *Myco. leprae.* This

decrease in ammonia has not been encountered with any other of the Mycobacteria studied.

In the mannitol broth column, then, there is one curve at each of the extremes, that is one showing no variation from the control (*Myco. friburgensis*), and a second curve (*Myco. leprae*) exhibiting definite variation from the control. The remaining curves occupy intermediate positions. The curves for *Myco. leprae* and *Myco. phlei* may be of value as a means of distinguishing these organisms from the other species studied. However, the large number of variable factors tending to alter the curves, such as amount of inoculum, amount of inoculum in contact with the surface of the medium, variability in the rapidity of growth from culture to culture etc., definitely limit the value of this distinguishing characteristic.

In the remaining two columns of curves, variations from the plain broth controls similar to those discussed in the mannitol broth cultures may be noted, that is in the presence of the carbohydrate there is in general a retarded reaction change. In the third column the curve for *Myco. friburgensis* in galactose broth is the only one closely simulating the curve of the plain broth control. Varying degrees of the diminished rate of pH change are to be noted in the remaining curves. The curve given for *Myco. chelonei* was obtained at a later date employing a different batch of media, there being very scant growth in the cultures used for the first determination. This fact possibly accounts for the variation from the other types of curves here encountered.

In the fourth column (glucose broth cultures) the curves of several of the organisms resemble closely the curves of their respective plain broth controls; for example, *Myco. tuberculosis*, *Myco. phlei*, and *Myco. friburgensis*. Other curves show merely a longer lag period before the alkalinity developed, e.g., *Myco. smegmatis*, *Myco. butyricum*, and *Myco. berolinensis*. The curve for *Myco. chelonei* constitutes a more definite variation. The growth was scanty and the results indefinite in the first cultures of this organism. The curve given in this case represents the results obtained at a later determination. The growth was more abundant in the latter cultures but even in this case there was

less growth than was obtained in the cultures of the other organisms given. This lesser growth probably accounts for the difference in the type of the reaction curve.

A secondary slightly diminished alkalinity is observable in the glucose broth culture of *Myco. leprae* which, like that appearing with this organism in mannitol broth, is irregular in occurrence. In this same column the curve for *Myco. stercusis* also presents a definite variation from the plain broth control.

The original object of these determinations was to ascertain whether or not mannitol, galactose, and glucose are utilized by the organisms studied. The reaction curves convey indefinite and conflicting evidence. Employing more accurate methods, which will be given below and in a subsequent report, all three of these carbon compounds have been shown to be utilized by all the organisms here studied, with the two exceptions that mannitol has not been shown to be utilized by *Myco. chelonei*, nor galactose by *Myco. leprae*. It must be remembered that the only evidence that carbohydrates are utilized obtainable from the curves is the decreased rate of production of alkalinity observable in some carbohydrate broth cultures. Many of the curves would suggest utilization of the respective carbon compounds; for example *Myco. tuberculosis*, *Myco. leprae* and *Myco. phlei* in mannitol broth; *Myco. tuberculosis*, *Myco. chelonei*, *Myco. smegmatis*, etc., in galactose broth; and *Myco. chelonei* and *Myco. stercusis* in glucose broth. On the other hand, there are not a few cases in which the curves present little or no evidence that utilization has occurred; e.g., all the curves for *Myco. friburgensis*, and several of the curves shown in the fourth column representing the results in glucose broth cultures. Glucose is especially actively utilized by these organisms, yet the curves in most cases indicate no such utilization.

It therefore must be concluded that the determination of reaction change in the media constitutes an indefinite and misleading method of determining carbohydrate utilization by these organisms. Such a method may, however, under carefully controlled conditions, be applicable for differentiation of some of the organisms here studied. The reaction curves obtained when *Myco.*

leprae and *Myco. phlei* were grown in mannitol broth and when *Myco. chelonei* was grown in glucose broth vary sufficiently from the remaining curves in the determinations here given to be of possible differential value.

The significance of these reaction curve determinations undoubtedly rests more upon its clear cut demonstration of the great similarity in the metabolism of the organisms of this group than upon their carbohydrate utilization. These organisms have already been shown to be nearly related (Long, 1923) first, in staining reaction; second, in the possession of a large amount of lipin; and third, serological reactions indicate that their antigenic activity is qualitatively the same. This work offers further evidence that they are very nearly related in their metabolic activities.

IV. DIRECT QUANTITATIVE DETERMINATION OF CARBOHYDRATE UTILIZATION

In order to obtain more exact information concerning the extent to which carbohydrates are utilized by organisms belonging to the genus under consideration direct quantitative determinations of the sugars were made.

Method

Standard broth, prepared as in the preceding work, was used as the base of the media. It was adjusted to pH 7.5 and the various sugars were weighed out and added in amounts to make approximately 1 per cent solutions. The carbohydrate-containing media were then immediately tubed measuring 10 cc. to each tube from burettes. Sterilization was by heat, at 5 pounds pressure for thirty minutes.

The inoculations were made as in the preceding work. The duration of incubation varied as shown in the accompanying table while the temperatures of incubation were as follows: *Myco. avium* 41°C.; *Myco. piscium*, *Myco. ranae*, *Myco. chelonei*, and *Myco. butyricum*, 22° to 25°C.; while the remaining organisms were incubated at 37.5°C.

The Shaffer-Hartman blood sugar quantitative method as recommended for bacteriological procedures by Stiles, Peterson and Fred (1926) was used for the carbohydrate determinations at the termination of incubation of the cultures. Before carrying out the tests for carbohydrates the pathogenic organisms were killed by immersing the cultures in a water bath at 100° C. for ten minutes. Each culture was then diluted to 100 cc. by pouring the entire contents of the tube into a 100 cc. volumetric flask, the culture tube being rinsed three times with distilled water to insure that all the sugar should be transferred into the flask. Following the addition of the lead acetate and potassium phosphate the flask was filled to the mark with distilled water, thoroughly shaken, a portion transferred to a test tube and then, after allowing fifteen to twenty minutes for the precipitate to settle, an aliquot portion of 1 cc. was withdrawn for each determination. Cultures were run in duplicate and two determinations were made with material from each culture.

The carbohydrates used were glucose, fructose, galactose, arabinose, lactose, and sucrose. The latter sugar was hydrolyzed prior to running the determinations by adding 1 cc. of concentrated hydrochloric acid to each tube, incubating at 70°C. for fifteen minutes, and subsequently neutralizing with concentrated sodium hydroxide using phenolphthalein as the indicator.

Results

The results of the main series of determinations are tabulated in table 1. In the first column under each sugar is given the duration of the incubation in days. In each of the next two columns is given the mean value of the two determinations from each culture tube. In the last column is given the average value obtained from the two culture tubes. The results are reported as per cent sugar in the culture media. The values given for the sugars, other than glucose, were arrived at by multiplying the amount of reducing sugar, in terms of glucose, as read from the tables given by Stiles, Peterson and Fred (1926), by the factors given in table 1.

TABLE 1

Determination of carbohydrate utilization by quantitative analysis

CULTURE	GLUCOSE*				FRUCTOSE* (FACTOR 1.0)				ARABINOSE* (FACTOR 1.14)				GALACTOSE* (FACTOR 1.14)				SUCROSE* (FACTOR 1.0)				LACTOSE* (FACTOR 1.65)			
	Incubation	Culture I	Culture II	Average	Incubation	Culture I	Culture II	Average	Incubation	Culture I	Culture II	Average	Incubation	Culture I	Culture II	Average	Incubation	Culture I	Culture II	Average	Incubation	Culture I	Culture II	Average
	days				*days*				*days*				*days*				*days*				*days*			
Controls:																								
Highest	16			0.88	18			0.82	19			0.74	20			0.76	30			0.94	28			0.82
Lowest	16			0.75	18			0.74	19			0.64	20			0.65	30			0.78	28			0.62
Average of five	16–38			0.81	18–38			0.78	19–38			0.70	20–38			0.72	30–38			0.88	28–38			0.78
Myco. tuberculosis (hominus)	16	0.13	0.19	0.16	18	0.07	0.09	0.08	19	0.07	0.07	0.07	20	0.12	0.10	0.11	30	0.80	0.86	0.84	28	0.70	0.78	0.74
Myco. avium	39	0.66	0.80	0.73	39	0.14	0.66	0.40	39	0.34	0.29	0.31	39	0.65	0.67	0.66	30	0.06	0.08	0.07	30	0.63	0.69	0.66
Myco. leprae 282	16	0.28	0.26	0.27	18	0.07	0.09	0.08	19	0.62	0.62	0.62	20	0.65	0.63	0.64	30	Trace			28	0.76	0.78	0.77
Myco. leprae 65	16	0.51	0.57	0.54	18	0.14	0.14	0.14	19	0.62	0.62	0.62	20	0.68	0.67	0.67	30	Trace			28	0.79	0.72	0.76
Myco. leprae 66	16	0.23	0.27	0.25	18	0.14	0.10	0.12	19	0.07	0.07	0.07	20	Trace			30	0.72	0.86	0.79	28	0.72	0.72	0.72
Myco. leprae 67	16	0.59	0.57	0.58	18	0.40	0.42	0.41	19	0.55	0.51	0.53	20	0.70	0.66	0.68†	30	0.85	0.73	0.79	28	0.72	0.86	0.79
Myco. leprae 68	16	0.36	0.40	0.38	18	0.07	0.09	0.08	19	0.07	0.07	0.07	20	0.41	0.37	0.39	30	0.08	0.26	0.17	28	0.79	0.73	0.76
Myco. leprae 69	16		0.16	0.16	18	0.04	0.04	0.04	19	0.00	0.00	0.00	20	0.00	0.00	0.00	30	0.80	0.86	0.83	28	0.72	0.74	0.73
Myco. piscium	38	0.56	0.42	0.49	38	0.49	0.21	0.35	38	0.67	0.62	0.64	38	0.78	0 75	0.77	38	0.89	0.89	0.89	38	0.74	0.66	0.70
Myco. chelonei	38	0.07	0.07	0.07	38	0.66	0.68	0.67	38	0.56	0.64	0.60	38	0.69	0.71	0.70	38	0.70	0.68	0.69	38	0.74	0.76	0.75
Myco. ranae	38	0.65	0.59	0.62	38	0.30	0.26	0.28	38	0.60	0.58	0.59	38	0.83	0.88	0.86	38	0.92	0.92	0.92	38	0.76	0.74	0.75
Myco. butyricum	38	0.68	0.78	0.73	38	0.59	0.57	0.58	38	0.38	0.40	0.39	38	0.19	0.17	0.18	38	0.82	0.78	0.80	38	0.76	0.76	0.76
Myco. smegmatis	16	0.08	0.08	0.08	18	0.05	0.05	0.05	19	0.00	0.00	0.00	20	Trace			30	0.86	0.88	0.87	28	0.79	0.71	0.75
Myco. berolinensis	16	0.20	0.19	0.20	18	0.08	0.08	0.08	19	Trace			20	0.07	0.14	0.10	30	0.76	0.82	0.79	28	0.70	0.72	0.71
Myco. phlei	16	0.28	0.28	0.28	18	0.07	0.07	0.07	19	0.00	0.00	0.00	20	0.12	0.12	0.12	30	0.91	0.89	0.90	28	0.65	0.65	0.65
Myco. stercusis	16	0.37	0.62	0.50	18	0.06	0.06	0.06	19	0.00	0.00	0.00	20	0.14	0.16	0.15	30	0.76	0.82	0.79	28	0.65	0.65	0.65
Myco. friburgensis	16	0.13	0.13	0.13	18	0.13	0.07	0.10	19	0.21	0.15	0.18	20	0.26	0.42	0.39	30	Trace			28	0.70	0.70	0.70

* All values represent the per cent of the respective sugars present in the media at the termination of incubation, determined as given in the report.

† More utilization demonstrated in subsequent tests.

The results demonstrate that glucose is utilized by all organisms studied. Utilization by *Myco. avium* is seen to be slight in the results listed. It should be noted, however, that the growth in these cultures was also slight. In subsequent tests greater utilization by this organism was demonstrable.

Fructose was also utilized by all organisms studied. The slight utilization shown by *Myco. chelonei*, again, may be attributed to the slight growth. It is worthy of note that there was, with few exceptions, a more rapid utilization of fructose than of glucose. The growth was also more abundant. This relationship has been repeatedly observed.

Arabinose was definitely utilized by most of the organisms. There was, however, but slight utilization by *Myco. leprae* 282 and *Myco leprae* 65. This has been rechecked several times with similar results. Greater utilization by *Myco. leprae* 67 was demonstrated in subsequent tests. Some, though slow, utilization by *Myco. piscium* has been shown by subsequent tests. More definite utilization by *Myco. chelonei* was demonstrated in later tests than appears from the table.

There was less tendency towards utilization of galactose. *Myco. leprae* 282 utilizes galactose very slowly. There is apparently no utilization by *Myco. leprae* 65. In later tests *Myco. leprae* 67 was found definitely to utilize galactose. Upon repeated tests, no utilization of galactose by *Myco. chelonei* and *Myco. piscium* could be demonstrated.

Utilization of sucrose is of especial interest. *Myco. avium*, the strains of *Myco. leprae*, 282,65, and 68 and *Myco. friburgensis* definitely utilize sucrose. There is apparently slow utilization by *Myco. chelonei*. It is possibly significant that the strains of *Myco. leprae* that utilize sucrose are rat strains whereas the strains of human origin do not utilize this carbohydrate.

Lactose is apparently not utilized by any of the organisms.

The summarized conclusions from these experiments as to definite, slight, and no utilization of the carbohydrates studied is given in table 2.

Some determinations have been made to ascertain whether maltose is utilized by any of these organisms. While the results

thus far are not entirely conclusive, definite utilization has been demonstrated only for *Myco. berolinensis*. These results clearly demonstrate a rather wide utilization of carbohydrates by organisms of the genus Mycobacterium. There is a distinct variation in the rapidity with which the various carbohydrates are utilized. In the course of the incubation periods adopted for this work, the carbohydrate contained in the media when utilized almost completely disappeared in many cases; in other cases only a slight decrease was noted. In general it appears that fructose is utilized more rapidly than any other carbohydrate studied.

TABLE 2

Summary. Carbohydrates utilized by organisms of the genus Mycobacterium

ORGANISMS	GLUCOSE	FRUCTOSE	ARABINOSE	GALACTOSE	SUCROSE	LACTOSE
Myco. tuberculosis (hominus)	+	+	+	+	0	0
Myco. avium	±	+	+	0	+	0
Myco. leprae 282	+	+	±	±	+	0
Myco. leprae 65	+	+	±	0	+	0
Myco. leprae 66	+	+	+	+	0	0
Myco. leprae 67	+	+	+	+	0	0
Myco. leprae 68	+	+	+	+	+	0
Myco. leprae 69	+	+	+	+	0	0
Myco. piscium	+	+	0	0	0	0
Myco. chelonei	+	+	+	0	±	0
Myco. ranae	+	+	+	0	0	0
Myco. butyricum	+	+	+	+	0	0
Myco. smegmatis	+	+	+	+	0	0
Myco. berolinensis	+	+	+	+	0	0
Myco. phlei	+	+	+	+	0	0
Myco. stercusis	+	+	+	+	0	0
Myco. friburgensis	+	+	+	+	+	0

The symbols have the following meaning: + indicates definite utilization; ± indicates slight utilization; 0 indicates no evidence of utilization.

These results also appear to demonstrate that carbohydrate utilization determinations are of limited value as a means of differentiation of the various species of this genus, since the variations observed between individual strains of the same species are in some cases greater than variations between different species of the same genus.

V. QUANTITATIVE RELATIONSHIP OF CARBOHYDRATE UTILIZED TO CARBON DIOXIDE AND AMMONIA PRODUCED, AND REACTION CHANGES IN THE MEDIA

Kendall, Day and Walker (1914) have demonstrated an increase of ammonia in cultures of some Mycobacteria. They stated that this increase was less in the presence of glycerol. No effort was made to correlate the ammonia production with carbohydrate utilization.

Novy and Soule (1925) have carried out extensive studies upon the respiratory relationships of various bacteria. The first study reported dealt with the human type tubercle bacillus. They found that the respiratory quotient, $\frac{CO_2 \text{ liberated}}{O_2 \text{ consumed}}$, of a culture of tubercle bacillus varied with the media upon which the organisms were grown. In glycerol media the R.G. value was 0.836. The theoretical value, were the glycerol alone broken down to carbon dioxide and water, is 0.857. When glucose agar was used the respiratory quotient as determined and corrected was 0.992. The theoretical value for glucose is 1.000. This is illustrated by the following equation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

These workers showed that, even in the presence of carbohydrates, the tubercle bacillus will not grow without oxygen. They also demonstrated that the growth is proportional to the oxygen supplied. It would thus appear that molecular oxygen is necessary before the tubercle bacillus can obtain energy from the carbohydrate. In other words, there is apparently no anaerobic cleavage of carbohydrates by this organism.

Novy and Soule did not attempt to correlate the amount of carbohydrate disappearing from the media with the carbon dioxide produced and the oxygen consumed.

Numerous quantitative determinations of end products of carbohydrate and protein bacterial metabolism have been made in connection with organisms other than the Mycobacteria, especially in recent years. In every case, so far as the author has

found, when the end products of carbohydrate metabolism have been studied some organic acids or alcohols have been found. Thus there has been encountered no report of complete utilization of a carbohydrate by a single species of bacteria, that is, complete cleavage of the carbohydrate to carbon dioxide and water. It is significant that in all such determinations facultative anaerobic organisms have been employed.

It is not clear what the end products of carbohydrate utilization are in the case of organisms of the genus Mycobacterium. In an effort to obtain some information concerning the nature of these end products, as well as to find an explanation for the characteristic reaction changes induced in the media by these organisms the following experiments were carried out:

First: Determinations were made of reaction change of the media, the change in ammonia content, the titratable alkalinity, the decrease in glucose, or fructose, and the carbon dioxide produced at the termination of a given period of incubation.

Second: These changes were followed, employing a continuous aeration method, at intervals of a few days on a single culture in order to determine whether the relationships found in the above work were maintained throughout the duration of incubation and under unchanging oxygen tension.

Only glucose and fructose were used for these determinations as previous work has shown that the metabolism is apparently similar regardless of the sugar employed.

Method

Meat infusion broth, prepared as stated above, again constituted the basis of the media. When glucose or fructose were used, sufficient was added to make a 1 per cent concentration. The medium was tubed accurately, 10 cc. per tube, employing a 10 cc. volumetric pipette. Sterilization of the media was by heat as in the previous experiment. Inoculation of the tubes was also as previously given.

Immediately after inoculation the tubes were placed in one liter Florence flasks, to which 50 cc. of approximately 0.2 normal barium hydroxide solution had just previously been added. The

flasks were immediately tightly stoppered using specially prepared rubber stoppers. Two or more flasks containing uninoculated controls were similarly prepared for each series. After three to four days incubation the majority of cultures had developed a firm pellicle. From this time on, the flasks were gently rotated at daily intervals to break the crust of barium carbonate which had formed on the surface of the hydroxide, in order to permit more complete absorption of the carbon dioxide formed. At the termination of incubation the flasks were shaken and placed at room temperature for twenty-four hours during which time they were again shaken several times, thus permitting complete absorption of the contained gaseous carbon dioxide.

Each flask was then opened and the amount of hydroxide that had been neutralized by the absorbed carbon dioxide was determined by titration to the disappearance of the blue color of thymolphthalein, employing 0.2 normal oxalic acid. The color imparted by thymolphthalein is intense and clearly visible against the white of the barium carbonate and barium oxalate. Thymolphthalein has the added advantage over phenolphthalein that the change of color is at a somewhat higher pH and as pointed out by Schollenberger (1928) this fact permits a more accurate titration of the carbon dioxide absorbed. The difference between the number of cubic centimeters of standard oxalic acid solution required to neutralize the hydroxide, in the culture flasks on one hand and in the control flasks on the other hand, represented the hydroxide neutralized by carbon dioxide. The number of cubic centimeters difference multiplied by 4.4 gave the milligrams of carbon dioxide absorbed by the hydroxide.

The broth culture was then diluted to 100 cc. in a volumetric flask. Duplicate carbon dioxide determinations employing the Van Slyke apparatus were then run in order to determine the carbon dioxide absorbed or retained by the media. Five cubic-centimeters of the diluted culture were used for each of these determinations. The diluted uninoculated culture was used as control; thus, the carbon dioxide contained in the distilled water used for dilution need not be taken into account since the difference between the control and culture determinations again repre-

sented the cubic centimeters of carbon dioxide contained in 0.5 cc. of undiluted media, or one-twentieth of the total carbon dioxide in the culture. Temperature and barometric corrections were made for each determination. The carbon dioxide thus determined plus that absorbed by the barium hydroxide represented the total carbon dioxide produced by the culture.

Other 10 cc. portions of the diluted media were used for pH determinations. Phenol red and thymol blue (alkaline range) were used as indicators and the pH determined colorimetrically. The cultures when diluted 1–10, as in this work, give a fairly accurate index of the pH as recently shown by Fawcett and Acree (1929).

Two 25-cc. portions of the diluted media were used, first, for the determination of the titratable alkalinity and, second, for the determination of the ammonia content. To each of the 25-cc. portions in N.P.N. tubes was added 0.25 cc. of 0.1 per cent phenol red. Employing N/40 sulphuric acid the number of cubic centimeters of acid required to bring the pH of the diluted culture to the same pH as the diluted uninoculated control was determined. This represented the increase in the titratable alkalinity of the culture.

The same 25 cc. portions were then employed for the ammonia determinations. Folin's aeration method was used, the air used being washed by passing it through sodium hydroxide and sulphuric acid solutions. One gram of sodium carbonate and 5 grams of sodium chloride were added to the diluted culture. The ammonia was collected in N/40 sulphuric acid. The sulphuric acid neutralized was subsequently determined by back titration with N/40 sodium hydroxide using a solution of methyl red, to which was added a small amount of methylene blue, as indicator.

The sugar determinations were run by the Shaffer-Hartman method as previously outlined, except that a protein free filtrate was not prepared. In several control tests it was found that more constant results were obtainable when no effort was made to remove the protein from the media by precipitation. This agrees with the results recently reported by Magee and Smith (1930).

In the interpretation of the results it must be borne in mind

that the values given are in most cases determined from comparison with the uninoculated controls. Since there were several of these for the work here reported it was deemed unnecessary to enlarge the table by their inclusion. The carbon dioxide determinations were controlled as given above. The milligrams of carbohydrate as determined in the uninoculated control tubes, minus the milligrams found in the cultures, gave the amount of carbohydrate that was utilized. The carbon dioxide possible to liberate from the carbohydrate was calculated by multiplying the milligrams of carbohydrate utilized by the factor 1.47, since 1.47 mgm. of carbon dioxide might be obtained from every milligram of a hexose which is quantitatively converted to carbon dioxide and water. Thus:

$$\underset{180}{C_6H_{12}O_6} + \underset{192}{6O_2} \rightarrow \underset{264}{6CO_2} + \underset{108}{6H_2O}$$

in which the figures represent one gram molecule of the various substances. Therefore, for every 180 grams of the hexose converted to water and carbon dioxide, 264 grams of the latter would be produced, or $\frac{264}{180}$, or 1.466 grams of carbon dioxide per gram of hexose. Then, in order to have a direct basis of comparison on a percentage basis between the carbon dioxide actually recovered and that which might possibly be produced from the hexose utilized, the per cent of the possible carbon dioxide which was recovered was determined by dividing the actual carbon dioxide by the possible carbon dioxide. Stated in equation form this relationship would be expressed as follows:

$$\frac{\text{Total } CO_2 \text{ recovered} \times 100}{CO_2 \text{ possible from carbohydrate utilized}} = \text{per cent of possible } CO_2 \text{ recovered}$$

Theoretically, if the hexose constituted the sole source of carbon, 100 per cent would be the maximum value possible if all the hexose were completely oxidized, but, since other carbon containing compounds are present, one might expect to obtain values greater than 100 per cent, provided the carbohydrate is converted quantitatively into carbon dioxide and water.

TABLE 3

Relationships existing between carbon dioxide production, carbohydrate utilization, pH changes, ammonia production, and changes in the titratable alkalinity of cultures studied

ORGANISM	MEDIA	CO_2 DETERMINATION			CARBOHYDRATE DETERMINATION			pH	NH_3 PER 10 CC.	NH_3 INCREASE PER 10 CC.	NH_3 INCREASE PER MILLIGRAM CO_2 PRODUCED	TITRATABLE ALKALINITY IN CC. N/40 H_2SO_4	TITRATABLE ALKALINITY IN TERMS OF MILLIGRAMS NH_3	LATTER MINUS NH_3 INCREASE	DURATION OF INCUBATION
		Absorbed	In media	Total	Utilized	CO_2 possible	Per cent of possible CO_2 recovered								
Section A															
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*			*mgm.*	*mgm.*	*mgm.*				*days*
Myco. tuberculosis (hominis)	G	71	8	79	52	76	104	7.6	2.8	1.3	0.016	3.2	1.4	0.1	7
	G	68	5	73	42	62	118	8.0	2.4	1.2	0.016	2.8	1.2	0.0	10
	F	106	9	115	60	88	130	8.0	3.8	2.9	0.025	3.6	1.5	−1.4	12
	P	26	7	33				8.4	3.0	1.5	0.045	4.0	1.7	0.2	7
	P	44	5	49				8.4	3.2	1.9	0.041	5.6	2.4	0.5	13
Myco. smegmatis	G	45	6	51	39	57	90	7.6	1.8	0.3	0.006	2.4	1.0	0.7	7
	G	66	6	72	44	65	111	8.0	2.4	1.2	0.016	3.0	1.3	0.1	10
	F	91	8	99	56	82	121	8.0	2.8	1.9	0.019	4.4	1.9	0.0	12
	P	23	8	31				8.4	2.8	1.3	0.042	4.0	1.7	0.4	7
	P	49	6	55				8.7	3.7	2.4	0.044	6.8	2.9	0.5	13
Myco. stercusis	G	40	3	43	21	31	139	8.0	2.5	1.5	0.035	3.0	1.3	−0.2	18
	F*	53	4	57	45	66	86	7.6	2.3	1.3	0.023	3.0	1.3	0.0	22
	P	26	4	30				8.2	3.0	2.0	0.066	5.2	2.2	0.2	21

Myco. berolinensis	G	45	4	49	33	49	100	8.0	2.1	1.0	0.02	3.2	1.4	0.4	10
	F	82	5	87	48	71	122	8.0	2.4	1.5	0.017	4.0	1.7	0.2	12
	P	46	7	53				8.6	3.7	2.4	0.045	7.6	3.2	0.8	13
Myco. phlei	G	48	3	51	21	31	164	8.4	2.4	1.4	0.027	3.8	1.6	0.2	18
	F*	87	4	91	71	104	87	7.6	1.8	0.8	0.009	2.8	1.2	0.6	22
	P	40	4	44				8.4	3.4	2.4	0.055	6.0	2.6	0.2	21
Myco. friburgensis	G	69	2	71	45	66	108	7.6	1.0	0.0	0.0	2.6	1.1	1.1	18
	F	133	3	136	80	118	115	8.4	1.4	0.4	0.003	4.0	1.7	1.3	22
	P	32	3	35				8.2	1.9	0.9	0.026	5.2	2.2	1.3	21
Myco. chelonei	G	50	2	52	43	63	83	7.3	1.9	0.9	0.017	0.8	0.3	−0.6	18
	F	80	4	84	50	74	113	7.6	5.4	4.4	0.052	2.6	1.1	−3.3	22
	P	22	1	23				7.6	4.2	3.2	0.14	3.2	1.4	−1.8	21
Myco. butyricum	G	11	1	12	6	9	133	7.1	1.7	0.7	0.058	0.0	0.0	−0.7	18
	F	97	3	100	67	98	102	7.7	1.4	0.4	0.004	2.6	1.1	0.7	22
	P	22	1	23				7.6	2.0	1.0	0.044	3.2	1.4	0.4	21
Myco. leprae 282	G	43	1	44	35	51	86	7.3	0.9	−0.1	0.0	0.6	0.3	0.4	10
	F*	66	3	69	55	81	85	7.2	0.6	−0.4	0.0	1.4	0.6	1.0	12
	P	15	1	16				7.6	1.7	0.7	0.044	2.4	1.0	0.3	13
Myco. leprae 67	G	26	2	28	13	19	147	7.8	1.5	0.5	0.018	2.4	1.0	0.5	18
	F*	90	2	92	74	108	86	7.2	1.0	0.0	0.0	1.2	0.5	0.5	22
	P	41	4	45				8.4	2.7	1.7	0.038	6.4	2.7	1.0	21

TABLE 3—*Concluded*

ORGANISM	MEDIA	CO_2 DETERMINATION			CARBOHYDRATE DETERMINATION			pH	NH_3 PER 10 CC.	NH_3 INCREASE PER 10 CC.	NH_3 INCREASE PER MILLIGRAM CO_2 PRODUCED	TITRATABLE ALKALINITY IN CC. N/40 H_2SO_4	TITRATABLE ALKALINITY IN TERMS OF MILLIGRAMS NH_3	LATER MINUS NH_3 INCREASE	DURATION OF INCUBATION
		Absorbed	In media	Total	Utilized	CO_2 possible	Per cent of possible CO_2 recovered								
Section B															
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*			*mgm.*	*mgm.*	*mgm.*				*days*
B. typhosus	G	4	0	4	26	38	10	<6.0	1.7	0.2	0.05	−7.6	−3.2	−3.4	7
	P	21	3	24				8.4	2.7	1.2	0.05	3.6	1.5	0.3	7
Staph. aureus	G	11	0	11	39	57	20	<6.0	1.9	0.4	0.036	−10.8	−4.6	−5.0	7
	F	6	0	6	36	53	11	<6.0	1.7	0.2	0.033	−12.4	−5.2	−5.4	7
	P	6	1	7				8.0	2.1	0.6	0.086	1.4	0.6	0.0	7
B. coli	G	10	0	10	40	59	17	<6.0	1.2	0.0	0.0	−10.4	−4.4	−4.4	7
	F	9	0	9	36	53	11	<6.0	0.9	−0.3	0.0	−11.6	−4.9	−4.6	7
	P	13	2	15				8.2	2.7	1.5	0.10	1.6	0.7	−0.8	7
B. subtilis	G	27	0	27	50	74	36	<6.0	1.5	0.3	0.011	−6.0	−2.6	−2.9	10
	F	29	0	29	20	43	67	<6.0	2.9	1.9	0.065	−4.8	−2.0	−3.9	12
	P	9	1	10				6.8	2.7	1.4	0.14	−1.2	−0.5	−1.9	13

Section C

Myco. tuberculosis (hominis)	1 per cent	G	173	11	184	88	129	142	8.6	5.1	4.1	0.022	6.4	2.7	−1.4	23
	2.5 per cent	G	308	9	317	212	310	102	8.0	3.2	2.2	0.007	4.4	1.9	−0.3	23
	3.5 per cent	G	284	9	293	221	324	90	7.6	1.4	0.4	0.001	3.2	1.4	1.0	23
Myco. smegmatis	1 per cent	G	165	12	177	85	125	141	8.6	5.1	4.1	0.023	6.4	2.7	−1.4	23
	2.5 per cent	G	277	9	286	211	311	92	8.0	2.5	1.5	0.005	4.2	1.8	0.3	23
	3.5 per cent	G	273	7	280	216	315	89	7.7	1.5	0.5	0.002	2.8	1.2	0.6	23
Myco. tuberculosis (hominis)	1 per cent	Gl	176	12	188				8.4	5.0	4.0	0.021	4.2	1.8	−2.2	24
	3 per cent	Gl	188	9	197				8.0	3.6	2.6	0.013	2.6	1.1	−1.5	24
	5 per cent	Gl	157	7	164				8.0	3.4	2.4	0.014	2.4	1.0	−1.4	24
Myco. smegmatis	1 per cent	Gl	176	14	190				8.4	4.0	3.0	0.016	4.6	2.0	−1.0	24
	3 per cent	Gl	127	1	128				7.4	3.3	2.3	0.018	1.2	0.5	−1.8	24
	5 per cent	Gl	134	1	135				7.4	3.3	2.3	0.017	0.4	0.2	−2.1	24

G = Glucose broth; F = fructose broth; P = plain broth; Gl = glycerol broth.

* These determinations were made immediately after removing the cultures from the incubator without allowing the usual 24-hour period to insure complete carbon dioxide absorption.

The expression "total ammonia per 10 cc." used in the table refers to the total ammonia found in the cultures, and the expression "ammonia increase per 10 cc." is this total, minus the ammonia found present in the uninoculated control. The ammonia present in the uninoculated controls varied in the different batches of media used for the reported determinations between 1.02 and 1.53 mgm. The expression "mgm. ammonia produced per mgm. carbon dioxide recovered" is self explanatory.

The titratable alkalinity is expressed in cubic centimeters of N/40 H_2SO_4 required to bring the pH of the culture to the pH of the control; this was determined colorimetrically. In order that a direct comparison may be made between the ammonia increase and the increase in the titratable alkalinity of the media the latter is also expressed in terms of milligrams of ammonia. This value, minus the determined ammonia increase, is given in the next column of the table.

In the control experiments in which non-acid-fast organisms were employed (*B. coli*, etc.) it was necessary to add alkali to bring the pH of the culture to that of the control. This is represented in the column giving titratable alkalinity by a minus sign before the number.

In analyzing the results the limits of the accuracy of the methods employed must be kept in mind. The following control experiments were designed to establish the degree of variation that might be expected.

Accurately weighed amounts of anhydrous Na_2CO_3 were added to 125-cc. Erlenmeyer flasks. These were placed in Novy jars to which a known amount of barium hydroxide had just previously been added. The cover was immediately placed on each jar and the jars partially evacuated by means of a water pump. By means of a tube, one end of which was attached to one outlet of the jar the other end being placed in the flask containing the sodium carbonate, an excess of sulphuric acid was allowed to run into the flask. The stop cocks were then closed and the flasks thus sealed from the atmosphere. After twenty-four hours incubation at 37.5 °C. approximately 85 per cent of the possible carbon dioxide was demonstrable from titration of the barium

hydroxide. After three days incubation 95 to 98 per cent of the carbon dioxide was recoverable. Similarly the Van Slyke gas analysis method was shown to be correct within 4 per cent by control determinations. Thus, the carbon dioxide determinations are, it may be assumed, correct within 5 per cent.

The sugar determinations are undoubtedly subject to a greater error. Upon control tests in which determinations were made on several uninoculated control tubes of glucose broth a maximum difference of 5 per cent between the lowest and highest values was found. This may thus be taken as the maximum limits of variation between parallel determinations; but, since the average value obtained by the Shaffer Hartman method is subject to at least 5 per cent error, a further possible variation must be taken into consideration. This last is demonstrated by the variations found in the controls throughout this work. Despite the fact that in making the media the glucose was accurately weighed and added in proportion of one gram made up to 100 cc. in volumetric flasks, values ranging from 0.95 to 1.06 per cent glucose were obtained by the Shaffer Hartman method. It should also be noted that the same reagents were used throughout these determinations, a sufficient quantity being prepared in the beginning for all tests. Thus, the maximum variation between the controls was approximately 10 per cent. It seems justifiable to consider this the normal limit of variation of the carbohydrate determination.

Colormetric methods were employed for the determinations of pH and of titratable alkalinity. Absolute accuracy was thus not attained in these determinations. The titratable alkalinity was determined upon but one fourth of the culture, so a variation of at least 0.4 to 0.8 cc. of N/40 acid might be expected to be encountered here. This represents 0.17 to 0.34 mgm. of ammonia.

Finally, since such a minute quantity of ammonia was present, the values obtained here may be stated to be correct only within 0.5 mgm. The widest variation found between all the controls on any one batch of media was 0.51 mgm. per 10 cc., or 0.14 mgm. for the 2.5 cc. of culture media actually used for the determination. This latter represents approximately 0.3 cc. of N/40 NaOH

variation in the actual titrations, since 0.1 cc. of N/40 NaOH is equivalent to 0.0425 mgm. of ammonia. Thus, the maximum error in any one direction of ammonia increase plus titratable alkalinity in terms of ammonia may be stated to approximate 0.85 mgm. Control determinations demonstrated the sodium carbonate, the sodium chloride, and the caprylic alcohol used as antifoam to be free of ammonia.

Results

Results typical of those obtained in a considerable number of determinations are given in table 3. In section A of the table the results obtained from 1 per cent glucose broth, 1 per cent fructose broth, and plain broth cultures of the Mycobacteria are given. A corresponding study in which non-acid-fast bacteria were employed is tabulated in section B. The results obtained when varying concentrations of glucose and of glycerol were added to the broth are reported in section C. The values given represent the average of the determinations upon two parallel cultures. Two such series are reported for the first two organisms listed in the table, when grown in glucose broth and in plain broth, for the purpose of demonstrating the variation normally encountered when the determinations are not made at the same time and when media from the same batch are not employed. In the other cases average results from one series of determinations are given. At least two series of determinations have been made for all the Mycobacteria reported except *Myco. chelonei* and *Myco. butyricum*. In some cases, e.g., *Myco. tuberculosis* and *Myco. smegmatis*, the experiments have been repeated three times.

Despite the limitations cited above to the accuracy of the methods employed, several significant relationships are clearly demonstrated. The following are the more important relationships apparent from data presented in Section A of the table:

1. In the majority of cases more carbon dioxide was produced in the carbohydrate broth cultures than in the control plain broth cultures incubated for approximately the same length of time. In the glucose broth culture of *Myco. tuberculosis*, 79 mgm. of carbon dioxide were produced in seven days; in the control plain broth culture, only 33 mgm. were produced in the same time.

2. The quantity of growth obtained may be stated to be roughly proportional to the carbon dioxide produced. No direct quantitative estimations of the amount of growth were made but it was observed that the bulk of the growth increased with increasing carbon dioxide production. Consequently a more luxuriant growth was obtained in the carbohydrate broth cultures of the Mycobacteria than in the plain broth cultures.

3. A significant relationship exists between the carbon dioxide recovered and that which could be produced from the carbohydrate utilized, were it quantitatively converted to carbon dioxide and water. It will be noted that, in the majority of instances, more carbon dioxide was recovered than could have been derived from the carbohydrate disappearing. For example in the glucose broth culture of *Myco. tuberculosis* 79 mgm. of carbon dioxide were recovered while 76 mgm. is all that could have been derived from the 52 mgm. of glucose utilized. In some cases, however, (e.g., the glucose broth culture of *Myco. smegmatis*, the fructose broth culture of *Myco. stercusis*, etc.) less carbon dioxide was recovered than that theoretically possible from the utilized carbohydrate. Seven such low values are given, but as noted in table 3 some of these low values are possibly attributable to the fact that sufficient time was not allowed after removal from the incubator to effect complete absorption of carbon dioxide. Most of these low values are within the limits of experimental error as noted above, while some values lie a little outside such limits. It is noteworthy, however, that in all determinations employing the two strains of *Myco. leprae* the per cent of possible carbon dioxide recovered has averaged less than for the other Mycobacteria studied; a single exception is in the results given with *Myco. leprae* 67 when grown in glucose broth. It should be mentioned that, wherever there is slight utilization, the possible error in the per cent of possible carbon dioxide recovered becomes magnified, since an error of as much as 10 mgm. in the glucose determination might possibly be encountered by the methods employed.

Since there is no definite evidence as to just how much of the carbon dioxide was derived from the protein constituents of the media it cannot be concluded from the above relationship alone

that the carbohydrates are completely oxidized to carbon dioxide and water by the Mycobacteria. These results, however, constitute important suggestive evidence that such complete oxidation of the carbohydrate does occur, especially when these results are compared with those reported in section B of the table.

4. The fructose appears to have been utilized more rapidly than the glucose by all the Mycobacteria studied. For example 42 mgm. of glucose were utilized in ten days by *Myco. tuberculosis* while 60 mgm. of fructose were utilized in twelve days. More striking is the utilization of 21 mgm. of glycose in eighteen days and 71 mgm. of fructose in twenty-two days by *Myco. phlei*. The slightly longer incubation period would not account for the differences observed.

5. There was an increase in the alkalinity of all cultures, which was less marked in the carbohydrate broth than in the plain broth. Thus, despite the fact that there was more growth and more carbon dioxide production in the carbohydrate broth cultures the degree of alkalinity was not as great as that found in the plain broth controls. In the seven-day glucose broth culture of *Myco. tuberculosis*, 79 mgm. carbon dioxide was produced incident to a change from pH 7.2 to 7.6 while in the plain broth control culture only 33 mgm. of carbon dioxide was produced incident to a change from pH 7.2 to 8.4. This retardation in the reaction change in the presence of utilizable carbohydrates was suggested in the reaction curves considered above; here it is definitely shown to exist.

6. Intimately related to the reaction changes, were the changes in the ammonia content of the culture media. The ammonia increase in the media was roughly proportional to the reaction change. No such absolute relationship, however, can be shown to exist. For example, when the various cultures had reached a pH of 7.6, values were obtained for ammonia increase ranging from 0.0 mgm. in the case of the glucose broth culture of *Myco. friburgensis* to 1.3 mgm. in the glucose broth culture of *Myco. tuberculosis*. However, the relationships existing between the ammonia increase and the increase in pH varied with the organisms tested, more ammonia being produced in all media tested

incident to the production of a given change in pH by certain organisms than by others. *Myco. friburgensis* produced little ammonia incident to a rather definite reaction change in the plain broth (reaction change pH 7.2 to 8.4; ammonia increase 0.9 mg.) as well as in the carbohydrate broth; conversely *Myco. chelonei* produced large amounts of ammonia with little associated reaction change (in plain broth pH change 7.2 to 7.6; ammonia increase 3.2 mgm.) and finally, in the glucose broth and fructose broth cultures of *Myco. leprae* 282, there was an actual decrease in the ammonia content of the media yet a slight increase in alkalinity was observed, at least in the glucose broth culture.

It is significant that, in every case, far more ammonia was produced in the plain broth culture per mg. of carbon dioxide recovered than in the carbohydrate broth cultures. In the glucose broth culture of *Myco. tuberculosis*, 1.3 mgm. of ammonia was produced incident to the production of 79 mgm. of carbon dioxide, or 0.016 mgm. ammonia per milligram CO_2, while 1.5 mgm. of ammonia was produded incident to the production of 33 mgm. of carbon dioxide in the plain broth control or 0.0440 mgm. ammonia per mgm. of CO_2. Thus, if we compare the ratio of milligrams NH_3 increase to milligrams CO_2 recovered we find almost 3 times as much NH_3 produced incident to the production of a milligram CO_2 in the plain broth cultures as in the carbohydrate broth cultures. A study of the column in table 3 giving the milligram ammonia increase per milligram carbon dioxide produced will demonstrate that a similar relationship exists throughout the Mycobacteria excepting in the case of the glucose broth culture of *Myco. butyricum*.

If the ammonia increase in the media may be taken as an index of the protein cleavage, a very distinct protein sparing action is here clearly demonstrated as a result of the presence of the carbohydrate.

7. Another interesting relationship is that existing between the titratable alkalinity and the increase in the ammonia content of the media. In most cultures the increase in the ammonia content of the media alone could account for the determined titratable alkalinity. However, this does not hold true in all cases.

For example there was no increase in the ammonia demonstrated in the glucose broth culture of *Myco. friburgensis*, yet there was a reaction change from pH 7.2 to 7.6 with a titratable alkalinity of the equivalent of 1.1 mgm. of ammonia. Likewise, there was an unmistakable decrease in the ammonia content of some cultures of *Myco. leprae*; yet the reaction changed toward increased alkalinity with a corresponding increase in the titratable alkalinity. In some tests this decrease in ammonia resulting from growth of *Myco. leprae* in the media was observed in plain broth cultures of this organism.

In several cases, e.g., the fructose broth culture of *Myco. tuberculosis*, all cultures of *Myco. chelonei*, and the glucose broth culture of *Myco. butyricum*, it will be noted that the titratable alkalinity (in terms of milligrams of ammonia) is distinctly less than the actual ammonia increase in the media. This indicates the production of compensatory acid products tending to neutralize the ammonia. This relationship has been observed in plain broth cultures as well as carbohydrate broth cultures and is constantly found after long incubation periods in either type of media, becoming progressively greater as incubation is prolonged, or in other words as protein cleavage increases. It is clearly shown in the plain broth culture of *Myco chelonei* given in table 3, and will again be referred to in connection with other cultures. Thus, the failure of the titratable alkalinity to be equivalent to the ammonia increase is in no way dependent upon the presence of carbohydrates. From the evidence at hand the conclusion therefore seems warranted that the changes in the ammonia content of the media account equally well for such reaction changes and changes in titratable alkalinity in carbohydrate-containing, as in carbohydrate-free, media.

8. From the above considerations it is apparent that there is no evidence that any cleavage products were formed from the carbohydrates that influenced the reaction change or change in titratable alkalinity. It would thus appear that the presence of the utilizable carbohydrate exerts an effect upon the reaction change of the media only to the extent to which it alters the protein metabolism, no cleavage products being formed from the carbohydrates themselves that tend to alter the reaction of the media.

By way of summary, then, it may be stated that the increase in the ammonia content of the cultures would account for all changes in pH and for the determined titratable alkalinity in the majority of cultures, whether in carbohydrate broth or in plain broth. In those case in which such is not the case it would appear that other cleavage products, which may be either acid or alkaline in nature, are produced which play some part in the production of changes in pH and in titratable alkalinity. These latter substances, however, appear to be derived solely from sources other than the carbohydrates since they are produced in plain broth cultures as well as in carbohydrate broth cultures; and furthermore the evidence at hand points to a protein origin since their production by a given organism approximately parallels the protein cleavage produced by that organism.

While the results obtained in this investigation point to a defiite similarity in the carbohydrate metabolism of the Mycobacteria it will be noted that there are apparently certain differences in the protein metabolism, as exemplified by differences in ammonia production, relation of ammonia production to change in pH, etc.

9. When we compare the results obtained from cultures of the Mycobacteria with those obtained from cultures of the four non-acid-fast organisms given in section B of the table, marked differences are noted. More carbon dioxide was recovered from the plain broth cultures than from the carbohydrate broth cultures of *B. typhosus* and *B. coli*. This contrasts strikingly with the results obtained with the Mycobacteria. Of special significance is the relationship of the carbon dioxide recovered to that possible of liberation from the carbohydrate utilized. Values ranging from 11 to 67 per cent recovery of the possible carbon dioxide with an average of 24 per cent emphasize the wide difference between the carbohydrate metabolism of these organisms and that of the Mycobacteria in which an average of 111 per cent of the possible carbon dioxide was recovered.

Also, the reaction of the carbohydrate media in the case of the non-acid-fast bacteria became definitely acid, a reaction more acid than pH 6.0 being produced in all cases. The titratable alkalinity, such as was observed in the cultures of the Mycobac-

teria, is here converted into a correspondingly much higher titratable acidity, for example, as much as 12.4 cc. of N/40 NaOH being necessary in the fructose broth culture of *Staph. aureus*, to bring the pH of the culture to that of the uninoculated control. As in the case of the Mycobacteria there is an increase in the ammonia content of the cultures of the non-acid-fast organisms. This increase is likewise greater in the plain broth than in the carbohydrate broth cultures.

The outstanding difference in the metabolism of the two general groups thus appears to be in the mode of utilization of the carbohydrates. In the case of the non-acid-fast organisms studied, there is obviously incomplete utilization of the carbohydrates with the accumulation of incompletely oxidized cleavage products in the media. This is the type of carbohydrate utilization that is demonstrable by the appearance of acid in the media. It is this type of utilization that has been most extensively studied and it is the only type that has been clearly defined in the literature the author has reviewed. It seems remarkable that the possibility of bacteria completely oxidizing carbohydrates to carbon dioxide and water has received such slight attention. The results given above indicate rather definitely that it is by such a process of complete oxidation, unaccompanied by the accumulation of any partially oxidized products in the media at any time, that the Mycobacteria utilize carbohydrates.

10. In order to determine whether the type of metabolism of the Mycobacteria is essentially the same when higher concentration of the glucose is present in the media, the experiments reported in the first half of section C of table 3 were carried out. Essentially the same relationship is here shown to exist between the carbon dioxide recovered to the amount that could be produced from the carbohydrate utilized as outlined for the Mycobacteria above. The high values for per cent of possible carbon dioxide recovered in the case of the 1 per cent glucose broth cultures might be attributable to the fact that practically all the carbohydrate had been utilized since, as will be considered below, the protein metabolism apparently increases as the carbohydrate concentration diminishes. Consequently in the low concentration

of carbohydrate present towards the end of the incubation the amount of carbon dioxide derived from the proteins would increase.

It might thus be suggested that the degree of protein-sparing action exerted by the carbohydrate is definitely dependent upon the number of molecules of that carbohydrate available for potential contact with the organism. It would appear not to be a case merely of the presence of the carbohydrate, (a given protein sparing effect being exerted so long as any of the carbohydrate molecules remain) but rather that the protein sparing effect progressively diminishes as the concentration of the carbohydrate diminishes. Striking evidence that such is actually the case is provided by a study of the effect of higher concentrations of carbohydrate on reaction changes, ammonia production, and titratable alkalinity as contained in the results given in section C of the table.

In the 1 per cent glucose broth cultures of *Myco. tuberculosis* and *Myco. smegmatis*, the terminal reaction was pH. 8.6 in both cases; a terminal reaction of pH 8.0 was found in the 2.5 per cent glucose broth cultures even though considerably more carbon dioxide was produced in these cultures than in the 1 per cent glucose broth cultures; similarly there was still less reaction change in the 3.5 per cent glucose broth cultures. There was a corresponding difference in the ammonia produced, progressively less being formed as the concentration of the carbohydrate was increased.

Thus, in spite of the greater amount of growth in the higher concentration of the carbohydrate as shown by greater carbon dioxide production, there was actually less ammonia produced, 4.1 mgm. increase in ammonia being noted in the 1 per cent glucose broth culture of *Myco. tuberculosis* incident to the production of 184 mgm. of carbon dioxide, as compared to 0.4 mgm. ammonia increase incident to the production of 293 mgm. of carbon dioxide in the 3 per cent glucose broth. This is especially emphasized by the values showing milligrams ammonia increase per milligram carbon dioxide recovered; for example in the cultures of *Myco. tuberculosis* these values are respectively 0.022, 0.007, and 0.001 for the 1, 2.5 and 3.5 per cent glucose broth cultures. A

pronounced increased protein sparing action with increasing carbohydrate concentration is here clearly shown.

The titratable alkalinity in terms of ammonia is less than the ammonia increase in the 1 per cent glucose broth cultures. This further emphasizes the fact mentioned above that with increasing protein cleavage, whether in plain broth or carbohydrate broth cultures, the ammonia increase becomes progressively greater than the titratable alkalinity as expressed in milligrams ammonia. The relationship here shown to exist between titratable alkalinity and ammonia increase in the 3.5 per cent glucose broth (1.0 and 0.6 with the *Myco. tuberculosis* and *Myco. smegmatis* respectively) further emphasizes the fact that, regardless of how much carbohydrate is utilized, there is no evidence that accumulation of products of the carbohydrate cleavage in any way alters the reaction or titratable alkalinity of the media.

In the second half of Section C are reported results obtained when glycerol replaced the sugars as an extra carbon source. The most significant finding here is that, in any of the concentrations of glycerol employed, much less carbon dioxide was recovered than from the 2.5 and 3.5 per cent glucose broth cultures. It would appear from these results that the addition of glucose in the latter concentration provides a better medium for the growth of these organisms than the addition of glycerol. This is contrary to the usual conception and is worthy of further investigation.

There is evidence of decreasing reaction change and ammonia production upon increasing the glycerol content of the media from 1 to 3 per cent as in glucose but not upon further increasing the concentration to 5 per cent. An increasing protein sparing action with increasing concentration of glycerol, as shown by the relationship of milligrams ammonia increase per milligram carbon dioxide produced, is not definitely shown from these results. Likewise, the ammonia increase is greater in relation to the titratable alkalinity than in the glucose broth cultures. Since the amount of glycerol utilized was not determined, the significance of this variation is not clear. From the amount of ammonia increase it would appear that this is a further manifestation of the production of compensatory neutralizing substances from the

protein constituents of the media. Summing up the results in the glycerol broth, it would appear that the same basic relationships are present as found in the carbohydrate media studied, the glycerol being completely oxidized to carbon dioxide and water.

VI. PROGRESSIVE CHANGES OCCURRING IN THE MEDIA WHEN THE CULTURE IS GROWN UNDER AEROBIC CONDITIONS

The results just reported were obtained from cultures which were grown under conditions which permitted of only a limited supply of atmospheric oxygen. The question arises as to what effect this gradually diminishing oxygen supply has upon the metabolism of the organisms. Novy and Soule have shown that the degree of growth of the human type tubercle bacilius is dependent not upon the oxygen tension but upon the total amount of oxygen available. Their work demonstrated that the oxygen could be used "to the last molecule" and that, thereafter, there were no further changes in the carbon dioxide relationships. There was no demonstrable carbon dioxide production after all the oxygen available had been used. Higher oxygen tensions permitted a more rapid growth but had no affect upon the respiratory quotient. It would appear from their work, therefore, that the gradually diminishing oxygen tension would have no affect upon the metabolic products formed.

What determinations the author has made thus far indicate that gradually diminishing oxygen supply in no way alters the relationships outlined in the preceding section. This work is being continued and will be reported in detail at a later date.

In order that the entire problem may be presented at this time, the results obtained from one of the experiments will be briefly summarized. Employing *Myco. smegmatis* five consecutive determinations were made both upon plain broth and glucose broth cultures; these were made just prior to inoculation of the media and after six, nine, fourteen and twenty days incubation.

A continuous aeration method was devised for these determinations. The method used was an adaptation, with certain modifications, of the methods reported by Krishna (1928) and by

Greaves, Zobell and Greaves (1929). A similar method has been just reported by Walker (1930). A water pump was employed for obtaining negative pressure. The incoming air was drawn successively through sodium hydroxide solution, sulphuric acid, sterile distilled water, the culture flask, N/40 sulphuric acid, and finally a carbon dioxide absorption tower. The latter was similar to that employed by Krishna while the arrangement of the culture flask with provisions for withdrawing specimens for analysis was similar to that used by Greaves *et al.*

By this method, it was possible to carry out all determinations upon the media reported in the previous section and, also, to determine the ammonia and carbon dioxide liberated from the culture.

During the entire incubation period 2023 mgm. of carbon dioxide were recovered from the glucose broth culture. At the termination of the third aeration period there remained no residual $Ba(OH_2)$ in the absorption tower, the available amount having been completely neutralized by the carbon dioxide. Some carbon dioxide was thus undoubtedly lost. Fifteen hundred milligrams of glucose as determined were utilized, from which 2190 mgm. of carbon dioxide could have been produced. Thus, despite the fact that there was some loss of the evolved carbon dioxide, 93 per cent of the carbon dioxide that could possibly be produced from the glucose utilized was recovered. Thus, the same relationships as were found under gradually diminishing oxygen tension apparently here also obtain, as concerns carbon dioxide output and carbohydrate utilized. Only 1635 mgm. of carbon dioxide were recovered from the plain broth culture.

The reaction of the media became progressively more alkaline in each case, more rapidly in the case of the plain broth culture. Both cultures were adjusted to pH 7.0 in the beginning. After 20 days incubation the reaction of the plain broth culture was pH 9.0 while that of the glucose broth culture was pH 8.6. At pH 8.4, which was reached on the sixth day in the plain broth and on the fourteenth day in the glucose broth, the ammonia content of the two cultures was the same, 1.7 mgm. per 10 cc. At pH 8.6, the ammonia content of the plain broth culture was 2.89 mgm.

per 10 cc., that of the glucose broth culture was 2.6 mgm. per 10 cc. This constitutes further striking evidence that the reaction change induced in the media by any given organism varies directly with the ammonia content, regardless of whether utilizable carbohydrate is present. It is also further evidence that it is presumably the derivatives of the nitrogenous elements of the media that are responsible for all reaction changes, the carbohydrate playing a part only to the degree that its presence alters protein metabolism.

A similar close comparison was noted in the titratable alkalinities of the two cultures. At pH 8.4, the titratable alkalinity of the plain broth culture was 2.1 mgm. NH_3 per 10 cc., while that of the glucose broth culture was 1.6 mgm. per 10 cc.; at pH 8.6, the values were 2.5 and 2.1 mgm., respectively.

In addition it may be mentioned that ammonia is given off by the culture. Recognizable quantities were not obtained until the cultures had reached pH 8.0. As the reaction became more alkaline, progressively more ammonia was liberated. In the first aeration period, during which the reaction of the plain broth culture changed from pH 7.0 to 8.4, 5.6 mgm. of ammonia were recovered; in the next aeration period of 3 days, during which the reaction changed from pH 8.4 to 8.6, 7.4 mgm. of ammonia were recovered; during the change from pH 8.6 to 8.8, 8.2 mgm. of ammonia were recovered; while 15 mg. were recovered in the final aeration period. It must be remembered that these values represent the total ammonia evolved from the 250 cc. of culture media under continuous aeration. The amount evolved from the 10 cc. cultures in the closed flasks reported in the previous section of this paper would, thus, be too slight to introduce appreciable error in those determinations.

VII. GROWTH OF ORGANISMS UNDER ANAEROBIC CONDITIONS

From the work of Novy and Soule and many previous investigators, the stringent oxygen requirements of the organisms belonging to the genus Mycobacterium are generally recognized. In order to determine whether this is a general characteristic of

all organisms used in this study attempts were made to grow the organisms under anaerobic conditions.

Glucose agar and plain agar tubes were inoculated, as well as glucose broth and plain broth tubes; these were incubated for twenty-one days in a phosphorous jar without the appearance of growth in any tube. Cultures of *Cl. tetani* in the same jar developed an abundant growth. After removal from the jar the same tubes, without further inoculation, were incubated under aerobic conditions with a resulting abundant growth by the end of one week.

In other experiments the oxygen supply was definitely limited. Test tube cultures of the saprophytic Mycobacteria placed in N.P.N. tubes and sealed from the atmosphere with rubber stoppers developed a moderate growth within two weeks. Some of the cultures were tested at this time for pH and for glucose utilization. Other parallel cultures were incubated for eight weeks more and then similarly tested. These latter cultures were found to contain approximately the same per cent of glucose and had the same pH as the cultures tested after 2 weeks incubation. Thus, despite the fact that these cultures had been subjected to 8 weeks further incubation, there had been no further carbohydrate utilization or reaction change. Many tests have been run in which the oxygen supply has been thus limited with the same results. Even if a good growth of the organisms has developed while the free oxygen is gradually decreasing there is no further utilization of carbohydrates or further growth after the oxygen supply has apparently all been exhausted. The organisms do, however, remain viable under anaerobic conditions as the first experiment given above illustrates.

Thus, it would appear that the organisms of this genus possess no ability to obtain energy from carbohydrates under anaerobic conditions. Most bacteria, e.g., *B. coli*, *B. subtilis*, etc., can obtain some energy from carbohydrates by anaerobic cleavage to lactic, acetic, and other acids or to alcohol and carbon dioxide. Organisms of the genus Mycobacterium are thus apparently placed in a group by themselves.

VIII. ATTEMPTS AT DEMONSTRATION OF ORGANIC ACID PRODUCTION BY THE MYCOBACTERIA

In an effort to demonstrate the production of organic acids *Myco. smegmatis* and *Myco. tuberculosis* (*hominis*) were inoculated into flasks of 1, 2.5, 5, 7.5, and 10 per cent glucose broth and into plain broth. After five days incubation of the 1, 2.5 and 5 per cent glucose broth cultures an effort was made to determine whether any organic acids were present. No evidence of such could be found by distillation. No test could be elicited for lactic acid in the ether extract using ferric chloride. After three weeks incubation, the 5 and 7.5 per cent glucose broth cultures were similarly tested, with no evidence of organic acids present.

Growth did not appear in the 10 per cent glucose broth culture until about the tenth day. Growth was then slow for the subsequent week, after which time the pellicle rapidly became heavier until by the end of one month a very heavy growth was present. No evidence of organic acids could be found from the tests upon these cultures. All cultures were more alkaline when tested than when inoculated.

The same tests were run on cultures which were subjected to a limited oxygen supply in an effort to force utilization. Twenty-five cubic centimeters of the respective media were placed in N.P.N. tubes. After sterilization, inoculations were made and the cotton stoppers pushed down a short distance into the tube and then the tubes were tightly sealed by inserting rubber stoppers. Upon incubation, a pellicle of approximately the same thickness formed on the surface of the media in every tube. The medium in all tubes was more alkaline at the termination of incubation than when inoculated. Again, no organic acids could be demonstrated.

Thus, if organic acids of any type are formed as intermediary products in the carbohydrate metabolism of these organisms they do not accumulate in the media. Also, there is no evidence of any ability on the part of these organisms to derive any energy from carbohydrates in the absence of molecular oxygen. In other words there can be no partial cleavage of carbohydrates by these organisms; apparently they oxidize the carbohydrate molecule

completely or not at all. The end result of carbohydrate utilization in animal organisms is carbon dioxide and water but if animal tissues are subjected to anaerobic conditions there is an accumulation of lactic acid. In the latter case molecular oxygen is, thus, not necessary for the primary cleavage of the carbohydrate. The author knows of no other example in animal or plant physiology where energy is derived from carbohydrates only in the presence of molecular oxygen.

IX. DISCUSSION

That the organisms belonging to the genus Mycobacterium do utilize some carbohydrates is clearly demonstrated by the present work. The demonstration of this fact, however, which was the object in view at the beginning of this investigation, is undoubtedly not the only significant aspect of this investigation. New light is thrown on many aspects of the metabolism of this group of organisms and possibly on the metabolism of other bacteria. Combinations of old methods have been used and some new methods have been introduced which are valuable in establishing general relationships in bacterial cultures. These perhaps may be of value if extended to a study of other groups of organisms.

The organisms of the genus Mycobacterium apparently have a singularly characteristic type of metabolism. It is due to this fact that some of the fundamental principles governing the carbohydrate metabolism of these organisms have been entirely overlooked. We have probably become too accustomed to considering acid production from carbohydrates as the criterion of fermentation or utilization. The more readily demonstrable type of utilization is of course such a type of incomplete cleavage of the carbohydrate. Here, the reaction progresses rapidly and comes to completion in the course of a few hours with inhibition of further growth of the bacteria, apparently because of accumulation of the cleavage products. In this type of utilization a large amount of carbohydrate is acted upon in a short time with the liberation of a relatively small amount of energy. For example, in the conversion of one gram molecule of glucose to lac-

tic acid approximately 19 calories of heat would be liberated (calculation from the heats of combustion given in Handbook of Chemistry and Physics, Ninth Edition, Hodgman and Lange). On the other hand, if the same amount of glucose were oxidized completely to carbon dioxide and water, approximately 670 calories of heat would be liberated. This is illustrated in the following equations:

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH + 19\ \text{Calories (approximately)}$$
$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 670\ \text{Calories (approximately)}$$

Approximately 35 times as much glucose would have to be attacked by the former method of utilization as by the latter method for the obtaining of the same amount of energy. Considered from this standpoint the explanation of the greater rapidity with which the former type progresses becomes clear. It is logical to expect that in the case of those organisms that oxidize the carbohydrate completely, as the Mycobacteria apparently do, there might be a relatively slow, though progressive decrease in the carbohydrate content of the media with a considerable production of energy. This is what has actually been found to occur.

Another significant factor is the rôle played by the per cent of the substrate present in the media. It was clearly pointed out by Kendall (1921) several years ago that carbohydrates exert a protein sparing action. The fact that this action is increased by increasing the concentration of the carbohydrate in the media does not seem to have been previously demonstrated. Possibly this phenomenon is encountered only in the case of the Mycobacteria but it seems possible that, if it occurs in this case, it may occur in other instances. The question of the concentration of the substrate is unquestionably of importance in certain industrial processes dependent upon bacterial action, as for example the manufacture of vinegar (Allen, 1926). If, in this process, the alcohol be allowed to fall below 2 per cent concentration the acetic acid bacteria begin oxidizing the acetic acid which they had previously been producing. The rôle played by the concentration of the substrate, whether it be the original substrate or a cleavage product which under proper conditions the organ-

isms can further oxidize, is a field that has been little investigated and that is rich in possibilities.

In view of the fact that the organisms of this genus have been found to be so closely related in their metabolic activities through the present study and previous studies reported by other workers it seems reasonable to expect that the respiratory relationships found for the tubercle bacillus by Novy and Soule are typical for the entire group. The present investigation, then, aside from demonstrating actual utilization of carbohydrates by the Mycobacteria has gone further in an attempt to correlate the respiratory relationships as established by Novy and Soule with various quantitative biochemical changes occurring within the media.

It is interesting that no inhibition to the growth of the Mycobacteria was noted in this work when the organisms were grown over barium hydroxide. According to the results of Rockwell (1921) such inhibition might be expected to be encountered due to the diminished carbon dioxide tension. The author's observations coincide more nearly with those of Novy and Soule (1928) who found no apparent inhibition to growth upon removal of carbon dioxide as completely as possible.

X. SUMMARY

1. The method of determining carbohydrate utilization by noting the appearance of acid in the media, which is the method generally used for such bacteriological determinations, is not applicable to the determination of utilization of carbohydrates by organisms of the genus Mycobacterium.

2. The determination of the reaction curves, using a modification of the method of Long and Major, is an indefinite and impracticable method of determining carbohydrate utilization by organisms of this genus.

3. The reaction curves produced when *Myco. leprae* is grown in mannitol broth, when *Myco. chelonei* is grown in glucose broth, and when *Myco. phlei* is grown in mannitol broth were possibly characteristic enough to be of value as methods of differentiating the strains of these organisms used in this work from each other and from the remaining members of the genus. The reaction

changes are, however, influenced to such a profound degree by slight environmental changes that even these atypical curves are considered too indefinite to be of absolute differential value.

4. The reaction changes in plain broth and carbohydrate broth that typify the organisms of this genus lead toward progressive increase in alkalinity. The reaction change is less rapid in the presence of utilizable carbohydrates.

5. There is a rather wide utilization of carbohydrates by the organisms studied, as demonstrated by direct quantitative carbohydrate determinations. The conclusions as to the carbohydrates utilized are given in table 2.

6. The increase in alkalinity, whether in plain broth or carbohydrate broth cultures, is associated with an increase in the ammonia content of the media. This increase in ammonia in most cases is approximately equivalent to the increase in the titratable alkalinity. In other cases, other substances, either acid or alkaline in character, which are apparently derived from protein cleavage, are involved to some extent in the causation of the reaction changes and the changes in titratable alkalinity. Such changes are accounted for by the ammonia increase, equally as well in the carbohydrate broth cultures as in the plain broth cultures.

7. The ammonia production was distinctly less for the production of a given amount of growth in the presence of utilizable carbohydrates. This diminished ammonia production was associated with a decrease in the degree of reaction change of the media. Also, the higher the carbohydrate concentration, so long as it is within the limits that will permit growth, the less the ammonia increase and the less the reaction change of the media during the production of a constant amount of growth. Thus, not only the presence but also the concentration of a carbohydrate is a factor determining the degree of protein sparing action it exerts.

8. The ammonia content of the media varied directly as the pH, the ammonia contained in the media being approximately the same at any given pH for the same organism in carbohydrate-containing as in carbohydrate-free media. However, there was

no constancy found in the ammonia content of the media at any given pH when different organisms were grown in the same media.

9. The presence of utilizable carbohydrate does not have any direct affect upon the reaction change of the media. Utilizable carbohydrates apparently affect the reaction changes only to the extent that they alter protein cleavage by the organism. Thus, there is no accumulation of cleavage products from the carbohydrate that in any way alter the pH of the medium. This contrasts strikingly with the carbohydrate utilization by most bacteria belonging to other genera.

10. The utilization of carbohydrates by the organisms of the group studied is characterized by a gradual decrease in the carbohydrate contained in the media until it completely disappears. There is, thus, no inhibition to the growth of the organisms at any time by products of the carbohydrate cleavage such as is the case in the majority of organisms the carbohydrate utilization of which have been studied.

11. The carbon dioxide produced has been shown to vary directly with the growth of the organisms, in carbohydrate-containing as well as carbohydrate-free media. The carbon dioxide produced also varied directly with the carbohydrate utilized in the carbohydrate-containing media, and in most cases more carbon dioxide was recovered than could have been derived from the carbohydrate disappearing. This may be compared to a 10 to 18 per cent yield of possible carbon dioxide recovered from cultures of acid-producing organisms each as *B. coli* and *Staphylococcus aureus*. Far more carbon dioxide was found to be liberated per milligram of ammonia produced in the presence, than in the absence, of utilizable carbohydrates.

12. No inhibition to the growth of these organisms was noted when the cultures were placed in jars containing barium hydroxide. Thus, if removal of carbon dioxide does exert inhibition to growth such removal was by the present methods not complete enough to exert any effect.

13. The organisms could not be grown under anaerobic conditions. Cultures grown in limited oxygen supply grew until all the oxygen was used and, thereafter, no further growth or carbo-

hydrate utilization or reaction changes in the media were demonstrable, even after two months' additional incubation. Carbohydrate utilization, thus, takes place only in the presence of molecular oxygen.

14. No acid cleavage products of the carbohydrate utilization could be demonstrated either in media of varying concentrations of carbohydrate or in the presence of varying quantities of available oxygen.

15. The explanation suggested in the preliminary report, namely that if the carbohydrate molecule is attacked at all it is oxidized completely to carbon dioxide and water, without the accumulation of demonstrable amounts of any intermediate products in the media, explains all the phenomena observed relative to carbohydrate utilization by these organisms.

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THE NATURE OF THE GRAM COMPOUND AND ITS BEARING ON THE MECHANISM OF STAINING

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Quite recently Burke and Barnes (1929) have brought up the question of the mechanism of the Gram reaction, and, reviving a theory rather ably supported by Benians (1920) have given data purporting to show that staining differentiation is due to what may be thought of as a selective sieve action on the part of the cell walls of various organisms in permitting large dye-mordant molecules to pass through. There seems no doubt that the existence of a large dye-mordant molecule is necessary if this is the mechanism. As stated by Burke and Barnes, "The cell wall theory assumes a change in the size of the dye molecules with the addition of the iodine or mordant." It will be the object of the present paper to present data which indicate very strongly that this theory is built on the assumption of a substance which is non-existent in any soluble form. It is true that in aqueous solution a precipitate of seemingly definite composition is formed. Experiments with standard dye and standard iodine show that the composition is one molecule of dye to two atoms, or one molecule of iodine. It seems equally true, however, that, in alcohol or acetone, this precipitate redissociates completely into iodine and the free dye.

EXPERIMENTAL

Two lines of experimental evidence are presented, a study of the absorption spectra of dye in the absence and presence of iodine, and a study of the boiling point change due to the solution of the dye-iodine precipitate.

A. Absorption spectra

When fluorescein is brominated eosin is formed. Solutions of both dyes are red in appearance with transmitted light. One might not expect any great shift in the maximum absorption band and yet this halogenation gives a shift of some 26 millimicrons. Now, if solutions, in either acetone or alcohol, of methyl violet and of the methyl violet-iodine precipitate are prepared, the former is a violet in color and the latter a rich red. If these

TABLE 1

Absorption spectra

SOLUTION OF DYE		SOLUTION OF DYE-IODINE PRECIPITATE	
Dilution	Wave length range	Dilution	Wave length range
	A. Alcohol solutions		
	mμ		mμ
1	612–532	1	612–528
2	603–541	2	608–538
3	598–578	3	600–571
4	591 (maximum)	4	591 (maximum)
	B. Acetone solutions		
1	636–497	1	626–516
2	624–511	2	618–528
3	612–532	3	617–525
4	607–578	4	609–544
5	593 (maximum)	5	593 (maximum)

colors are due to differences in absorption of two different compounds we should expect a shift in the methyl violet band much greater than 26 millimicrons. Table 1 gives readings on the methyl violet band for both solutions of pure dye and of dye iodine precipitate.

If there is a shift at all in the methyl violet band it is certainly less than 1 mμ. The change in color in the visual appearance of the solution is due to the superimposed absorption of the free iodine which absorbs in the short wave region. This can be shown qualitatively by placing a solution of precipitate next to

one of pure dye so that they may be compared. Now, by interposing a separate solution of iodine between the light source and the pure dye one can easily duplicate the color of the solution of precipitate.

B. Boiling point elevation

While the above experiment is almost conclusive it may be argued that halogenation might take place with no shift in absorption, though it seems to the authors that it is virtually impossible for two different pure compounds, the one giving a violet and

TABLE 2

Boiling points

Solvent: Acetone

TIME	THERMOMETER READING (CORRECTED)	
10:40 a.m.	2.141	Pure acetone
11:15 a.m.	2.139	
11:40 a.m.	2.138	
1:55 p.m.	2.139	
1.00 grams of Dye-I_2 precipitate introduced at 2:00 p.m.		
2:15	2.167	The slow rise is due to time required to completely dissolve the sample
2:35	2.195	
2:50	2.221	
3:20	2.223	
3:45	2.223	
4:20	2.222	

Final volume—85 cc., boiling point rise = 0.082°

the other a red solution, to have nearly identical absorption bands if the one is chemically derived from the other. Coupled with the following data, there seems no doubt of the dissociation of the compound into free dye and iodine, at least in acetone.

In this experiment a fairly high degree of precision is required since the molecular weights of both dye and iodine are so high that relatively small boiling point changes are obtained at practicable concentrations. Thus, it is necessary to know the boiling temperature to within 0.002° to 0.003° and this necessitates a precise knowledge of any changes in barometric pressure to

0.1 mm. during the course of an experiment, and correction therefore.

A Cottrell boiling point apparatus was finally employed. This insures equilibrium between solution and vapor without variations due to changes in hydrostatic head met with where the thermometer bulb is placed in the liquid.

A sample of precipitate was prepared and this was washed free from free iodine by KI solution and finally washed free from iodides by water, and then dried.

Preliminary experiments indicated that the elevation in the boiling point caused by alternate additions of dye and iodine or of iodine and dye are additive but these data are not included since it might well be argued that the compound must be formed in water to be sure of reaction. In table 2 are given data on the final experiment.

The apparent molecular weight is calculated from the formula:

$$M = K\frac{W}{e\,v} = 2220 \times \frac{1.00}{0.082 \times 85} = 319$$

Here K is the volume boiling point constant for acetone, w is the weight of the solute, e is the boiling point elevation and v the volume solution.

On the basis of an undissociated compound the molecular weight should lie between 641 and 674, depending on whether the dye used was the tetra, penta, or hexa methylated compound. Complete dissociation should result in an apparent molecular weight of 320 to 337. The experimental value was 319. This value also shows that the solute cannot be in any large colloidal state under these conditions.

The results of these experiments seem to the authors, when taken in conjunction, to indicate conclusively that no new compound of any stable nature is formed between dye and iodine. In aqueous systems the two mutually precipitate each other, and, while it is beyond the scope of this paper to inquire into the nature of the precipitate, analogous phenomena can be cited. The one which immediately comes to mind is the case of the system quinone hydroquinone which, when present in sufficient quantity,

precipitates as sparingly soluble quinhydrone, an equimolecular mixture of the two, but which dissolves as quinone and hydroquinone.

DISCUSSION

At first sight one of the chief points of strength in the argument of Burke and Barnes (1929) is the fact that they seek to differentiate what they term the "true" Gram reaction from what they call pseudo-Gram reactions, the two having essentially different mechanisms. Thus they specifically delimit the problem of rationalizing the results of a particular technic. The present authors are in complete agreement with them that, as a rationalization of the results of the Gram technic *alone* their postulated mechanism is entirely satisfactory, though they feel it is not the true one. They believe that the actual mechanism of this reaction cannot be illuminated, much less discovered, by confining one's attention to this technic alone. Organisms they believe are not in this manner excused from obeying fundamental laws of behavior which they are known to obey under very similar, if not identical, conditions.

Even without the experimental results presented here, the type of cell wall theory usually given runs into difficulty in relation to general staining behavior. To give only one example, staining with dyes not precipitated with iodine should not, on the basis of this theory, be affected by its use. Yet as a matter of fact, the use of iodine *decreases* the retaining power of organisms for certain acid dyes (Stearn and Stearn (1924, 1925)). While this is contrary to the sieve picture it is easily explained on the mechanism given previously by the present authors (1924b). Let us picture what happens to the cell wall theory in its ordinary form on the basis of the present data. It is admitted that the dye molecules pass through any cell wall readily, as do those of iodine and decolorizer. The dye is added to a smear and a "pseudo-Gram reaction" occurs (i.e., combination between protoplasm and dye, the mechanism which the present authors consider the chief factor in the retention of stain. It is significant that Burke and Barnes admit the possibility of this mechanism as a primary re-

action.) Iodine is now added and the dye protoplasmic compound decomposed by precipitation of the dye. Decolorizer is finally added and the dye-iodine precipitate is dissolved, but not as a new compound, rather as free dye and iodine. Now, if differential decolorization depends on the presence of large molecules trying to pass a membrane, then all organisms should be decolorized, since the free dye molecules should be able to pass out through the membrane through which they entered. The authors have already shown that it would require the addition of over 14 atoms of iodine to double the molecular volume, which would result in an increase of only 13 per cent in molecular diameter. The symmetrical addition of 2 atoms of iodine, representing the composition of the precipitate, would result in only a 2.5 per cent increase in diameter. They have, therefore, never seriously considered this change in size even before they knew of the fact that the compound did not exist in solution in the decolorizer; and undertook the present experiments only in the search for a type of evidence which is independent of any appeal to quantitative molecular volume considerations. There must, therefore, be some *other* factor which is effective in retaining dye, even in the "true" Gram reaction.

In this connection a preliminary qualitative experiment is of interest. Slabs of gelatin were stained with methyl violet at varying pH's. Some were treated with iodine and some not. At low pH's those treated with iodine were, as Burke and Barnes would predict, more easily decolorized than those untreated. At high pH's this was not the case. The freshly decolorized slabs which had had iodine treatment seemed at first almost opaque. After some hours they appeared to undergo a change giving a distinctly deeper blue than the unmordanted slabs. Here, there can be no question of cell wall. Alkali would not be expected to harden the surface layer as it tends to dissolve the gelatin, forming sodium gelatinate. With unsaturated materials such as soaps of unsaturated acids, the pH range through which iodine increased dye retention was wider. Cases were found where the unmordanted gelatin slab would take the counterstain and where, with the exception of the time element, one slab would

be called "truly" Gram-positive and the other Gram-negative. It is true that these substances are not protoplasm but such results as these should be impossible on the form of the cell wall theory of Burke and Barnes.

The announcement by Churchman (1929) of a reversal of Gram reaction by pretreatment with basic dye and the postulation of a cortex layer has been taken as giving support to the cell wall theory. From a study of the plates he presents it can be easily seen that it cannot support the type of cell wall theory postulated by Burke and Barnes. As soon as the cortical layer is even partly gone the organism should become completely negative. Yet there can be partial reversal in which certain areas retain stain while others do not. These experiments are comparable in certain respects to some reported by the present authors (1929) in which it was shown that starving bacteria *gradually* lose Gram positivity passing through a stage where they present a stippled appearance with Gram-positive granules throughout. Many of these starved cells show the same diminution in size reported by Churchman. Now, if these results are to be reconciled with a cell wall theory, it will be obviously necessary to postulate a very complex network of walls throughout the cell and if this is done it becomes difficult to reconcile the findings of Burke and Barnes that expression of endoplasm from cells cause both endoplasm and ectoplasm to become Gram-negative. On the other hand, it is perfectly reasonable that a fairly prolonged pretreatment with dye might tend to leach out those components with which the dye most easily combines and which the present authors consider the important factor in dye retention in the Gram stain (or any other staining technic of similar character; i.e., wherein the mechanism is not one of reduction as in silver or osmic acid staining), and thus, when the Gram technic is subsequently employed, varying degrees of reversal would be expected as noted by Churchman. We are thus in letter, if not in interpretation, in agreement with Churchman's statement that the Gram reaction "rests on an anatomical basis," but in the sense that a change in anatomy, especially when brought about by chemical treatment or starving, is hardly dissociated from an alteration

not only in the constitution of the organism as a whole but even in that of specific regions. The prevalent notion that chemical theory necessitates the idea that "the cell wall has nothing to do with the reaction" (Burke and Barnes (1929)) may confuse the issue. It is in the sense of a chemically inert sieve membrane that the present authors feel the cell wall cannot be effective. This point has been discussed more completely elsewhere (Stearn and Stearn (1928)).

One must also never lose sight of the significance of the fact that the pseudo-Gram reaction of Burke and Barnes, which is admittedly chemical, gives essentially the true Gram classification (Scales (1922)). That is, the mordant, while technically necessary for Gram technic, is not essential for a closely parallel classification. It seems stretching the probability of pure coincidence that this should be the case if the mechanisms are entirely dissimilar, depending on whether iodine is present or absent. Even if one admit this last assumption, the validity of which is incompatible with the data presented in this paper, he would be driven directly to the conclusion that under a cell wall theory the wall permeability parallels the chemical constitution of the organism in a rather startling manner.

SUMMARY

1. The cell wall theory of the Gram reaction mechanism in its ordinary form necessitates the existence in the decolorizer solution of large dye-mordant molecules.

2. Absorption spectra indicate the presence of only free iodine and free dye in solutions of methyl violet-iodine precipitate in both alcohol and acetone.

3. The boiling point elevation of a solution of methyl violet-iodine precipitate in acetone indicates complete dissociation into iodine and dye.

4. These findings indicate a high degree of improbability for the ordinary cell wall mechanism for the Gram reaction.

5. They are in no way contradictory to the chemical mechanism formerly proposed by the authors.

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RENNIN PRODUCTION BY BACILLUS PRODIGIOSUS

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In 1892–1893 I published three papers (Gorini, 1892, 1893a and 1893b) in which I described the discovery that *B. prodigiosus* produces a rennin or chymase, viz., a milk-clotting substance or enzyme; and I arrived at the following conclusions, which at that time were entirely novel:

1. Clotting of milk by cultures of *B. prodigiosus* is independent of the cell content of the culture, since it is produced by the sterile cell-free filtrates of cultures after passage through a Chamberland filter.

2. Rennin production by *B. prodigiosus* takes place not only in milk but also in diverse types of media, even in the absence of casein and lactose (broth, gelatin, agar, potato).

3. Prodigiosus rennin does not appear to be a product of cell autolysis, since the clotting power of the cell free cultures does not increase with their age.

4. Prodigiosus rennin acts also upon heated and even upon autoclaved milk.

5. Prodigiosus rennin is highly thermostable, being destroyed only by heating to 100°C. for over fifteen minutes.

With respect to points 4 and 5, prodigiosus rennin appears to differ from calf rennin.

Two years ago, J. G. Wahlin published a paper (1928) which reaches the same results but without recognizing fully my results of thirty-six years before. Wahlin says only "Gorini (1893) found that *B. prodigiosus* possessed a rennin-like action in milk."

I am gratified that my results have found such new and complete confirmation after thirty-six years; but I feel that my priority should not be ignored.

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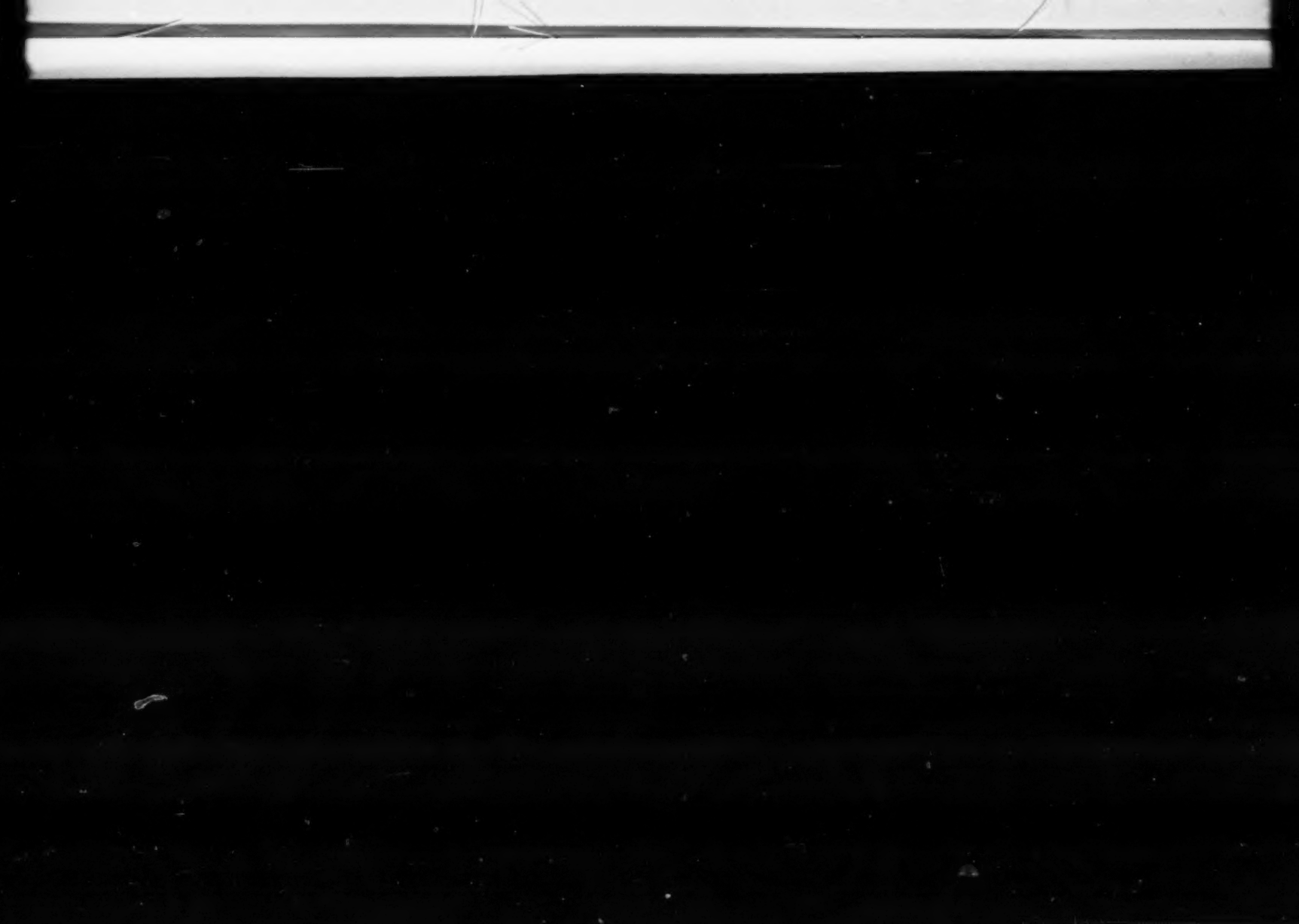

BACTERIAL SPORES

I. A STUDY IN HEAT RESISTANCE AND DORMANCY[1]

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Our knowledge regarding the properties of bacterial spores, *per se*, has been impeded by the more or less universal impression that spores are metabolically inactive or inert. Except for occasional theories concerning spore-cycles and the like, the usual conception of spore function has been limited to *ipso facto* sporulation under conditions of unfavorable environment, and germination when conditions again become favorable. Some exceptions are noted in the cases of certain organisms having peculiar tendencies toward dormancy or latent germination.

Spore properties, such as minimal metabolism, respiration, enzyme production and the possibility of varying degrees of pre-germination stimulation have received little attention or credence. There have been isolated attempts to gain information concerning these still hypothetical properties; some of these will be discussed later.

Just as studies of the intimate details of metabolism have aided greatly in other fields of biological research, so a finer comprehension of spore properties and spore function should result in material improvement and greater precision in studies of the heat resistance and dormancy of bacterial spores, a subject which so vitally affects various routine laboratory procedures and their industrial applications.

The two strains of the organism with which all of the preliminary work reported here was concerned were isolated in pure cul-

[1] This communication is based on a portion of a thesis submitted to the Graduate School of Yale University by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

ture from spoiled cans of evaporated milk from two widely separated sections of the country. These strains were considered as identical or of extremely close relationship, for certainly in all of their major characteristics, particularly resistance to heat, and in the type of spoilage produced in milk, they were alike. These organisms are non-thermophilic and produce a non-acid, non-gaseous coagulation or solid curd formation in the sealed cans or tubes of evaporated milk, totally different from the so-called flat-sour spoilage caused by thermophiles, which is so commonly encountered in various canned foods, including milk.

Although the type of spoilage produced is unusual, the occurrence of this organism in cans of milk from divergent sources suggested that this invading type is a common one, or as will be intimated later, perhaps a common dissociant of one of the ordinary aerobic, spore-forming species. In many of their characteristics these strains conformed to the descriptions of *B. vulgatus*, but, on account of dissociative tendencies, could not be positively identified as such.

LABORATORY DETERMINATION OF HEAT RESISTANCE

The heat resistance properties of the spores of the spoilage organisms were studied at the outset in order to obtain, if possible, data of immediate practical value in prescribing a safe sterilization temperature and time for factory use, in view of the fact that the spoilage obviously has been due to under-sterilization. For this reason also evaporated milk was used as the heating menstruum in the preliminary experiments.

SPORE SUSPENSION PREPARATION

Uniform, viable spore suspensions free from clumps and cellular débris are basically essential to many, if not all, forms of spore study, and more particularly experiments on resistance to heat. After investigation of several methods for the preparation of suspensions, the following procedure was devised and adopted because of its simplicity and reliability.

Plain nutrient agar slants, pH 6.8 to 7.0, are used in the cultivation of the organism. Incubation is continued until maximum sporulation

occurs. With the organisms under discussion, holding for two weeks at room temperature results in the presence of abundant free spores. The growth is washed from the agar surface with distilled water, and the crude suspension filtered through a cotton plunger tube to free it from débris and clumps. One filtration usually results in a surprisingly uniform, clump-free suspension. The cotton plunger tube consists of an ordinary test tube having a compact, closely fitting plug of non-absorbent cotton about one-third of the way down from the usual plug. The suspension is introduced below the plunger by means of a capillary-tipped pipette which is passed through or along the side of the cotton plug. The plunger is forced down through the suspension evenly with the pipette which is used for withdrawing the spore material. The spores are washed two or three times in distilled water by centrifugation, and finally suspended in phosphate-buffered distilled water, heated at 80°C. for ten minutes and stored in the ice box as the stock suspension.

Phosphate-buffered distilled water

NaH_2PO_4	4.0 grams
K_2HPO_4	6.0 grams
Distilled water	1000 cc.

This double phosphate solution has a pH value of 6.8 to 7.0 which does not change on heating in ordinary soft glass tubes unless the temperature is extremely high or the heating prolonged.

Except for certain modifications, the more or less standard method for heat resistance determination suggested by Bigelow and Esty (1920) was used in this work.

After determining the approximate range of the resistance of the spores in evaporated milk at the temperature employed (115.5°C.) a number of series of sealed tube vials were exposed to heat in the oil bath and tubes were removed at intervals of one minute within this range. Subcultures were made in standard nutrient broth and on standard agar plates. Agar plates were found to be most reliable in the detection of surviving spores.

Both of the spoilage strains, when heated in evaporated milk at 115.5°C., showed a survival time of fifteen minutes, and both were killed in sixteen minutes in numerous tests. All of the tubes in these series which were heated for shorter periods than the maximum survival time of fifteen minutes gave prompt growth in

the subcultures. Likewise, the killing was 100 per cent in tubes heated for longer periods than the survival time of fifteen minutes.

Immediately after completing these tests in the laboratory, experimental sterilization studies were undertaken in one of the milk canning factories. The technic used in this work was briefly as follows:

Cans of evaporated milk were taken from the track conveyor at the filling machine before being soldered. In the plant laboratory 0.5 cc. quantities of the stock spore suspension that had been used in the preliminary tests were introduced into the cans through the small vent hole, immediately before complete soldering. Extensive series of such inoculated cans were subjected to various sterilization temperatures and times

TABLE 1

Showing survival of spores as determined by visible spoilage and cultural tests

STERILIZATION TEMPERATURE	STERILIZATION TIME*	TIME OF INCUBATION AT 37°C.	PHYSICAL APPEARANCE	CULTURAL EXAMINATION
°C.	*minutes*			
110.0	30	24–48 hours	Curd; spoiled	Positive
115.0	15	24–48 hours	Curd; spoiled	Positive
115.5	15	24–48 hours	Curd; spoiled	Positive
116.0	15	48–96 hours	Curd; spoiled	Positive
116.5	15	3 months	Normal	Negative

* Exclusive of calculated time for heat penetration, as in the case of times reported for laboratory determination.

in the regular and in the sample sterilizers. After the required time of exposure the cans were cooled by turning cold water into the sterilizer. They were then labelled and stacked in the 37°C. incubator room.

The results of these sterilization experiments are presented in table 1.

It is interesting to note the close correlation between the results of the preliminary laboratory determinations of heat resistance and those obtained in the experimental sterilization work at the plant, the maximum survival temperatures being 115.5° and 116.0°C. for fifteen minutes respectively.

DISSOCIATIVE TENDENCIES OF THE SPOILAGE ORGANISM

When first isolated, both strains of the organism appeared on agar slant cultures as flat, even, dull, powdery gray growths

which were characteristic and constant in repeated transplants. Within three months, however, particularly in cultures over three weeks old, the appearance on agar had changed. Erosion or semi-lysis was apparent. Agar slant growths disappeared almost completely, leaving clear areas or a filmy secondary growth. A constant observation was that of papillate, secondary colonies superimposed on the agar surface growth in the older cultures. These colonies were smooth, white, glistening, opaque and raised.

The morphology of the bacterial cells in these colonies was at decided variance with that of the usual dull, powdery gray growth on the same medium. Filamentous forms, varying in size and shape and characterized by profuse irregularity in staining, were seen. Some of these stained deeply, some lightly, while others presented pictures of mottled, granular or banded light and dark staining. Also, depending upon the time of examination, regular rods and sporangia could be seen, varying from normal to forms approaching the filamentous stage.

This apparent dissociation was not of a stable or permanent nature, however, since transplants made from these raised, papillate colonies to fresh agar invariably resulted in immediate reversion to the rough, dull gray type of growth.

Limited attempts were made to obtain stabilized dissociants through the usual procedures of plating old broth or peptone water cultures, phenol-broth, or "R"-exhausted-broth cultures. Although these attempts failed to induce permanent dissociation, the possibility of obtaining stabilized forms by more intensive methods was recognized. However, a comprehensive study of dissociation is not within the scope of this work, although the tendencies of these organisms toward dissociation suggest that this phenomenon, particularly the possibility of deriving super-resistant "roughs" from common strains of the aerobic spore-forming bacilli, may play an important rôle in the bacteriology of food preservation.

Since the appearance of these dissociative tendencies persisted in all subsequent transplants, some attention was given in the further experimental work to the possibility of alteration or reversion of the organisms employed with respect to heat resistance.

To obtain direct evidence on this point, spores of the two strains were heated in sealed tube vials of evaporated milk, as in the original determinations, six months after the first tests. The resistance was found to be the same as it was at the outset.

DORMANCY OF THE HEATED SPORES

In further work on the heat resistance of the spores of the spoilage organisms, observations were made pertaining to the conditions under which the spores exhibited properties of dormancy or delayed germination after heating. In this work spore suspensions prepared in phosphate-buffered distilled water, as

TABLE 2

Showing heat resistance of spores in water, and dormancy of the heated spores incubated at 37°C. in nutrient broth

TEMPERATURE HEATED	TIME HEATED	TIME OF GROWTH IN DAYS																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	30	45	60
°C.	*minutes*																		
115.5	1	−	−	−	−	−	−	+											
115.5	2	−	−	−	−	−	−	−	−	−	−	−	−	+					
115.5	3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+		
115.5	4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+		
115.5	5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
115.5	etc. to 10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
115.5	15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

described previously, containing approximately 500,000 spores per cubic centimeter, were sealed in thin-walled tube vials in 1 cc. quantities and subjected to heat in the oil bath at 115.5°C. Tubes were removed from the bath at one minute intervals, subcultured in standard nutrient broth (Difco-Bacto meat extract and peptone) and incubated at 37°C.

The data given in table 2 were based on the average duration of dormancy in a number of tests, since in the various individual trials irregular results were obtained. "Skips" occurred, as described by Esty and Williams (1924), and by other authors, and variable periods of dormancy were noted in the subcultures of different tests. In some instances the results of heat resistance

tests in water, with sub-cultivation in plain broth, indicated that the resistance of the spores was lower than that shown in tests made only a short time before. These results seemed offhand to refute the earlier observations on the properties of the same spores heated in milk, in which no "skips" occurred and in which the thermal death time was sharply defined and apparently constant. Such would have been the conclusion were it not for the fact that parallel or confirmatory tests in milk revealed that the spores

TABLE 3

Showing the variable response and dormancy of the spores in plain nutrient broth following heating in buffered distilled water at 100°C. Incubation at 37°C.

TEMPERATURE HEATED	TIME HEATED	TIME OF GROWTH IN DAYS																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
°C.	*minutes*																				
100.0	0	−	−	+																	
100.0	1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+			
100.0	5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+					
100.0	10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+					
100.0	15	−	−	−	−	−	−	−	−	−	−	−	−	+							
100.0	20	−	−	−	−	+															
100.0	25	−	−	−	−	+															
100.0	30	−	−	−	−	−	−	+													
100.0	40	−	−	−	−	−	−	−	−	+											
100.0	50	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+					
100.0	60	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+			

Note: Critical point not determined at 100°C.; see page 305.

possessed their original resistance and showed no "skips" or dormancy when the heated milk suspensions were subcultured by plating out on plain nutrient agar.

Since the range of resistance of the spores heated in water at 115.5°C. was limited (maximum four minutes) a series of tests was run at a lower temperature to determine whether or not these idiosyncrasies were retained by the spores when the heating was not so severe.

It will be seen from table 3 that the same sort of irregular response is obtained at 100° as at 115.5°C., even though the maximum or critical time for the spores at 100° was not approached.

The occurrence of irregular results and variable periods of dormancy in all heat resistance tests on this organism in which the spores were heated in water and subcultured in plain nutrient broth, while regular results and no dormancy were obtained in tests in which milk was used as the heating menstruum and standard agar in plates as the subculture medium, suggested that the dormancy in the first instance was due to unfavorable environment, that is, that some factor, nutritional or otherwise, is lacking in the standard nutrient broth medium.

TABLE 4

Showing the action of the spoilage organisms as compared with that of other aerobic spore-forming bacteria

ORGANISM	DEGREE OF CLEAR ZONE FORMATION	TIME OF COLONY APPEARANCE	TIME OF CLEAR ZONE APPEARANCE	COLONY SIZE	MAXIMUM WIDTH OF CLEAR ZONE
		hours	*hours*	*mm.*	*mm.*
B. cereus	+	7	9	1–2	1
				4*	None*
B. subtilis	0	10			
B. vulgatus	+	9	12–16	3	1
				5*	None*
Spoilage str. I	++++	10	10	0.5	2.5
				1.5*	4.5*
Spoilage str. II	++++	10	10	0.5	2.5
				1.5*	4.0*

* Colony size and clear zone width at twenty-four hours.

THE ENZYMIC MECHANISM OF THE ACTION OF THE SPOILAGE ORGANISM ON MILK

The apparent predilection of the spoilage organism for milk, as evidenced by its prompt development in milk, even after severe heating, led to the following experiment, which was designed to gain some information regarding the nature of the action on milk.

A modification of the auxanographic method used by Eijkman (1901) in the study of bacterial enzymes was employed. Milk agar was pre-

pared by adding whole or evaporated milk to standard plating agar. Dilutions of the washed spore suspensions of the spoilage organisms, as well as of other forms, calculated to result in well-isolated colonies, were plated out on the milk agar and incubated at 37°C.

Around the colonies of the spoilage organisms clear zones appeared in the opaque milk agar, indicating that some agent capable of producing changes in the milk had diffused out from the colonies. Frequently these zones extended for a distance many times greater than the diameter of the colony itself. Table 4 indicates the action of the spoilage organisms as compared with that of other aerobic, spore-forming organisms.

It is noteworthy that with the milk spoilage strains the appearance of the clear zone is simultaneous with the appearance of the colony, while with *B. cereus* and *B. vulgatus* (laboratory strains) the colonies reach considerable size (two to seven hours growth after their first visible appearance) before the slightest clear zone can be detected. Also, the clear zones formed by the milk spoilage organisms not only appear earlier but are much more extensive and persist longer than do those produced by the other forms.

It is quite evident from these data that the spoilage strains produce secretions of an active substance, probably a caseolytic enzyme, that is at least partly accountable for the avidity of these strains for milk.

GENERAL DISCUSSION

Some of the observations made in the work reported here are at variance with previous findings and generally accepted theories concerning spore behavior.

Contrary to the present findings, variability in spore resistance to heat has been reported by numerous investigators, including Weiss (1921), Dickson et al. (1922), Esty and Williams (1924), Magoon (1926) and others. In general the view of most of these authors is that resistance is not a fixed property in any case, but an extremely variable one, being influenced by a host of conditions. Some are inclined to the view that there are wide variations in the resistance of individual spores in a single suspension.

In view of the complexity and multiplicity of factors that have been reported from time to time as influencing resistance, it would be difficult indeed to offer an explanation of the apparent constancy in resistance observed by us in the spores of the spoilage organisms when uniform suspensions were heated in evaporated milk and incubated, with or without subcultivation on agar. The marked variability shown by spores from the same suspensions when heated in water and subcultured in standard nutrient broth, suggests that evaporated milk in this instance supplies a consistently favorable, protective and uniform environment, which, together with the maintenance of other desirable factors, results in reducing any tendency toward variation to an absolute minimum

The writers' views are in accord with those of the above mentioned authors in so far as recognition of the possibility of variation in spore resistance is concerned, since it occurred in this work under certain conditions, and because variability is a law of living things. On the other hand, the opinion is offered that the extreme variability so often reported for the spores of various organisms is associated with conditions that are unfavorable or extreme, either during storage of the stock spore suspensions, or in the course of testing, or in the subculture environment. Just as there are conditions which tend to favor or induce variability in the resistance of spores, so there are also conditions that influence or bring about constancy of spore resistance.

Similarly, the absence of "skips" in single tube series of heat resistance tests on spores of the spoilage organisms, when the heating menstruum is evaporated milk and the subculture medium is agar, must be attributed to the favorable nature of nutritional and other factors in the environment, for the reason that with changes in the heating and subculture mediums to water and standard nutrient broth, respectively, duplicate sets of spore suspensions from identical stocks exhibited "skips" in the majority of instances.

In any discussion of "skips" and "killing range" in heat resistance determinations the uniformity of the spore suspensions of course cannot be ignored. In this work emphasis has been

placed upon the importance of this factor and on the heating of sufficiently large numbers of spores in each tube of a test series to further obviate unequal distribution of the spores

The phenomenon of dormancy or delayed germination of aerobic spores after heating has been frequently reported as occurring in environments entirely favorable for the germination of the spores in question. Magoon (1926), for example, reported that spores of *B. mycoides* remained dormant in subcultures (heat resistance tests) for periods varying from one to sixty-four days. He attributed this dormancy to normal, inherent properties within the spores, rather than to "heat inhibition" or injury due to heat, which has been suggested by Burke, Weiss and others as the cause of dormancy after heating. Neither of these views offers an explanation for the dormancy observed in the plain standard nutrient broth subcultures in the heat resistance tests on the spoilage organisms under consideration here. Both theories are untenable in this case, for the reason that with a change in pabulum from plain broth to milk the dormancy is eliminated. This fact limits the cause of the dormancy in this instance to environmental factors.

There is considerable evidence, direct and indirect, in support of the view that dormancy of heated or unheated spores is a function of the environment; in other words, that it is dependent on stimuli supplied by the environment. Further experimental work bearing on various environmental factors, particularly nutrition, in relation to dormancy, and discussion of this subject, will appear in a later communication.

A brief discussion concerning the function of nutrition in the germination of spores of the spoilage organisms under consideration is included here, since a certain amount of experimental evidence has been presented to show the mechanism of the action of these organisms on milk. It has been indicated that the peculiar adaptability of these organisms to milk may be due in part to the predominance of enzymes elaborated by them which are capable of attacking certain materials present in milk. It is not a far cry, then, to presume that the effectiveness of milk in stimulating

germination of the spores after heating is due to the presence of like enzymes in the spores, themselves, and that the secretion of these enzymes takes place prior to the actual germination.

The objection to such an hypothesis will be raised that enzymes are thermolabile and therefore can have no part in the rapid development of the spores in milk after heating to the extremely high temperatures mentioned. Granting this objection, the fact remains that the vital, life forces of the spores have been in no way impaired by the heat, as evidenced by their prompt germination. It is conceivable, then, that the enzymes may be regenerated by the spores, perhaps at an unusually rapid rate due to the stimulation of the temperature shock. The view, in this instance, that enzyme production and secretion precedes and facilitates germination by rendering the substrate utilizable by the germinating spores, is in direct confirmation of the work of Ruehle (1923) and that of Effront (1917) concerning the enzymic content of bacterial spores. The following statement of Effront is particularly apropos in this case: "Bacterial spores attenuated either by heat or antiseptics show themselves the more productive of enzymes the more difficult their germination. Under certain conditions and in the presence of antiseptics the spores may produce an intense secretion of enzymes in a liquid without, however, arriving at germination."

The suggestion has been advanced that the spoilage organisms are possibly super-resistant varieties of some common aerobic, spore-forming organism because of the tendencies toward dissociation. In this connection particular interest attaches to the work of Kelly (1926) on bacteria causing spoilage of evaporated milk. He has observed three types of spoilage, and it is significant that he designated the causative agents as atypical strains of *B. cereus*, *B. simplex* and *B. megatherium*, respectively. His results are highly suggestive as a possible confirmation of the contention that spoilage may be due frequently to super-resistant variants of common forms. In the writers' experience, ordinary strains of *B. cereus* and *B. megatherium* are notably lacking in resistance to heat.

SUMMARY

Observations made in a study of the properties of a heat-resistant, aerobic spore-forming organism suggest that the response or behavior of bacterial spores after heating is dependent upon environmental conditions, and that what appears to be extreme variability in spore resistance to heat is due to the lack of specific favorable conditions in the culture medium rather than to inherent qualities of variability.

Spores of the milk spoilage organism in question gave regular results, without dormancy or delayed germination, in heat resistance determinations when evaporated milk was employed both as the heating and subculture medium, but extremely irregular results were obtained when nutrient broth was used as the subculture medium. It is significant that this organism is peculiarly adapted in its enzymic function to the environment supplied by milk.

Because of certain dissociative tendencies, this spoilage organism was thought to be a relatively stable variant of a common aerobic spore-forming species, *B. vulgatus*, which it resembles in many ways.

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BACTERIAL SPORES

II. A STUDY OF BACTERIAL SPORE GERMINATION IN RELATION TO ENVIRONMENT[1]

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Perty (1852) was probably the first to observe and describe bacterial spores. Pasteur (1870), incidental to his work on diseases of the silk worm, confirmed the observations of Perty, finding similar elliptical, light-refracting bodies in bacteria. He extended the knowledge concerning the characteristics of these bodies by observing that they possess much greater resistance to injurious agents than do the bacterial cells themselves. Cohn (1877) was the first to establish the true nature of these bodies as spores, since Perty and Pasteur both failed of proof, not having observed the actual processes of sporulation and germination. Cohn saw the appearance of the light-refracting spore within the cell and observed the process of germination of spores of *B. subtilis* directly under the microscope.

Ever since this earlier work bacterial spores have been the basis of controversies as to their formation and function. That there are spore forms in bacteria other than the classical endospore has been claimed by numerous investigators, particularly in connection with the hypothesis that spores function as reproductive bodies. Hueppe (1885) described swollen, irregular elements in old cultures of several organisms, which he termed "arthrospores." Conidia formation was observed by Lister (1873) in cultures of *B. lactis*, and later by others in various organisms. These forms often were regarded as spores, and in fact Enderlein

[1] This communication is based on a portion of a thesis submitted to the Graduate School of Yale University by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(1925), who has reported them in many bacterial species, considers them as analogous to the ascospores of the fungi, and indeed as the true bacterial spores. Mellon (1925) described forms occurring in bacteria of the colon-typhoid and diphtheria groups, which he called "zygospores" because of their complete parallel to isogamic conjugation or zygospore formation in yeasts. Nocard and Roux (1887), Metschnikoff (1888), Wherry (1913) and others have observed bodies within the tubercle bacillus which resembled ordinary spores.

Numerous theories have been evolved concerning the causes and conditions, both cellular and environmental, leading to spore formation. One of the first was that of Buchner (1890), which designated exhaustion of nutrient material as the primary factor. Preisz (1904) considered sporulation as a definite stage in the development of bacilli, and held that the optimum conditions for the initiation of the process are identical with those favoring maximum vegetative development. Matzuschita (1902) recognized lack of nutrient material as a factor, but attached more significance to oxygen accessibility in sporulation. He stated that aerobic bacilli under hydrogen or vacuum of less than 30 mm. never form spores. Hall (1922) found that sporulation of the anaerobic bacilli was most abundant in mediums characterized by a low content of fermentable carbohydrates, such as deep brain, alkaline egg broth and blood agar. Henrici (1924) showed that spore formation is initiated at the end of the active growth stage of a culture and that, due to this fact, sporulation proceeds more rapidly in quarter strength medium because of the earlier cessation of active growth. Churchman (1925) attacked the conception that sporulation is merely a protective mechanism brought about by the stimulus of adversity in the environment, and presented a certain amount of evidence from the standpoint of dye inhibition of sporulation in support of his contention. He stated that certain members of the tri-phenyl-methane series of dyes, among other effects, have the property of inhibiting rather than inducing sporulation. The complexity of the phenomenon of sporulation was further indicated in the work of Daranyi (1927), who attributed the principal rôle to colloidal influences. His

explanation was that upon aging a shrinking of the cell colloids occurs which brings about the stimulation necessary for the initiation of spore formation. He drew an analogy between bacterial cells and the body cells of animals, pointing out that age in the latter is characterized by a diminution of the water content and a concentration of the colloids. In support of this contention he presented evidence that sporulation could be induced in young, actively growing bacteria by diminishing the cell water content by artificial means.

Any review of the literature concerning the germination of bacterial spores must embody material of diversified nature, for this property of spores is intricately involved in studies on lag, dormancy, heat relationships, single spore isolation, disinfection, bacterial nutrition, and the rôle of bacterial spores in disease. Preisz (1918) contributed a critical discussion on the finer structure of bacterial spores and changes incident to germination. By means of a delicate, intra-vital staining technic, using dilute fuchsin solutions, he was able to observe the cytological changes occurring in the process of germination, which he described and illustrated in detail. Meyer (1909) brought out the relationship of spore germination to oxygen. His tables evaluate the minimum, optimum and maximum concentrations of oxygen for the germination of spores of numerous species. For example, expressed in amounts of oxygen per liter, he found that with *B. subtilis* 4.3, 400 and 4317 mgm. represented respectively the minimum, optimum and maximum. Eijkman (1918) reported that the germination of spores of *B. anthracis* was hindered by the presence of salts that raise the osmotic pressure of the culture medium. He found that sodium chloride, sodium nitrate and levulose prevented germination at approximately the same osmotic pressure, as determined by the freezing point method, although the respective concentrations were greatly different.

Direct observation of the germination time of bacterial spores was undertaken by Swann (1924). There had been a dearth of investigation on this subject, in spite of its obvious practical applicability, probably due to the fact, pointed out by Swann, that it had been taken for granted generally that spores placed in

a favorable environment would germinate promptly. In his direct microscopic observations he made distinction between two types of spores to be found under different cultural conditions, the "young type" and the "old type." He found that the germination time of the "young type" of anthrax spores was short (1 hour and 30 minutes), constant, and independent of the age of the spore, while that of the "old type" was variable (minimum two hours and maximum seven hours). Allen (1923), in work concerned with the attenuation of bacteria due to temperature shock, brought out a point that was contradictory to the general trend of opinion in regard to the rôle of injury in latent germination or dormancy. He found that with spores of *B. subtilis* more rapid germination and growth were exhibited after a heat shock of 145°F. for thirty minutes than before.

There has been some confusion in the usage of the terms "lag" and "dormancy" or delayed germination. However, Burke and his collaborators (1925) evaluated the terms satisfactorily, designating "lag" as the latent period of a few hours shown by transplanted cultures, while "dormancy" expresses the prolonged quiescence of a few cells in the transplant after the majority have multiplied. Although dormancy or delayed germination of bacterial spores has been observed repeatedly and encountered as a disturbing factor in various bacteriological studies, there has been no concerted effort to get at the underlying causes of this phenomenon until comparatively recent years, perhaps for the reason that dormancy has been more or less universally accepted as an unavoidable natural phenomenon.

The classical example of dormancy, of course, is that so frequently observed with spores of *Cl. botulinum*. Burke (1919, 1923), Dickson *et al.* (1925), Dickson (1927) and numerous others have reported extensive observations and investigations on the spores of this organism. The prevalent note in this work has been that dormancy is inherent or normal in the spores of *Cl. botulinum*, as contended by Burke (1923) in her work with unheated spores, and that dormancy is accentuated in heated spores, due to the injury and shock caused by the heat.

Burke, Sprague and Barnes (1925) initiated a further develop-

ment in the study of dormancy. They presented evidence to show that the phenomenon of dormancy, usually associated with the spores of *Cl. botulinum*, is exhibited by the spores of common aerobic organisms, and indeed by non-sporing bacteria. Under conditions stated to be optimal for growth, they found that cells of *E. coli* remained dormant for as long as sixteen days and that unheated spores of *B. subtilis* and *B. megatherium* were dormant for thirty-nine and ninety days, respectively. This they spoke of as "normal dormancy."

That dormancy may be encountered as a disturbing factor in various researches is well illustrated in the work of Barber (1920) on the single spore isolation technic. Isolated spores showed variable germination times and Barber was inclined to attribute the instances of delayed germination to slight but significant differences in tubes of medium from the same batch. Starin (1924) also encountered dormancy following single spore isolations, finding that, although the majority of the positive cultures appeared within a week, a small percentage of the single spores exhibited prolonged periods of dormancy, reaching ninety days.

In the rôle of dormancy in disease, emphasis has been placed on the question of latent tetanus infections. Canfora (1907), Koser and McClelland (1917), and others have recognized the possibility of latent infection being caused by spores lying dormant in the blood or organs, particularly in case of traumatic injury or secondary invasion by other organisms in the immediate vicinity of these spores, creating conditions favorable to germination.

EXPERIMENTAL

In a previous communication (1930) the authors made certain observations pertaining to conditions under which spores of a given thermo-resistant aerobic organism exhibited properties of dormancy. The bacillus in question was isolated from spoiled cans of evaporated milk, the spoilage being due to understerilization. In the course of heat-resistance determinations with this organism, it was found that spores heated in buffered distilled water and subcultured in standard nutrient broth showed marked

and variable periods of dormancy. In parallel tests on spores from the same suspension heated in evaporated milk, with or without subcultivation on agar, the dormancy was completely eliminated. This observation suggested that the dormancy in the first instance was neither "heat induced" or "normal" dormancy, but rather an expression of the inadaptability of the spores to the particular environment supplied by the standard nutrient broth. The variability of these spores in their germination time after heating, depending upon the nutritional or other stimuli supplied in the environment, led to the following investigations which were designed to shed some light on the extent to which various environmental factors influence the germination, and hence the dormancy, of washed unheated as well as heated bacterial spores.

1. The germination time of aerobic spores as influenced by the nutritive value of the culture medium

In preliminary attempts to determine the effect of various culture mediums on germination two criteria of germination were adopted; the one was a macroscopic study of the extent of growth in a given time, and the other a determination of the increase of viable cells, by the interval plate counting method. Throughout this work washed spore suspensions were used, and efforts were made to keep inoculums uniform in all comparative tests.

The culture mediums employed in this work were made up as follows:

a. The plain nutrient broth was prepared from Difco Bacto meat extract and peptone according to standard methods of the American Public Health Association Committee.

b. The tomato-extract peptone medium was prepared according to the method of Kulp (1927) and had the following composition:

1 part filtered juice of canned tomatoes
2 parts distilled water
1 per cent peptone
Reaction adjusted to pH 6.8

c. Various forms of yeast autolysate or yeast extract mediums have been in use since the time of Pasteur (1858). As prepared in this laboratory the medium consists of an extract (autolysate) of yeast (Fleisch-

mann's) obtained by incubating 1 pound of yeast in 5 liters of distilled water for 3 days, with a small amount of ptyalin, and sufficient chloroform to prevent bacterial growth. This concentrated extract is steamed, filtered, clarified and sterilized by autoclaving. The finished medium consists of:

1 part concentrated extract of yeast
2 parts distilled water
1 per cent peptone
Reaction adjusted to pH 7.0
(Optimal amounts of glucose may be added)

d. Glucose broth was prepared from the standard nutrient broth base by the addition of desired amounts of glucose.

e. Sugar-free broth was obtained from the standard nutrient broth by the removal of traces of fermentable sugars by *E. coli*.

f. The synthetic medium consisted of the following ingredients:

$(NH_4)_2SO_4$	0.3	gram
$MgSO_4$	0.07	gram
KH_2PO_4	0.1	gram
K_2HPO_4	0.016	gram
NaCl	0.05	gram
$Ca(NO_3)_2$	0.04	gram
H_2O (distilled)	100	cc.
Glucose	0.5	per cent

As shown in table 1, the tomato-extract and yeast-extract mediums had a definite stimulating influence on the germination of washed unheated spores of the aerobic organisms, as compared with the plain, sugar-free, and to a certain extent with the glucose broth. Spores of the various organisms exhibited marked differences in their responses to the deficient mediums. A "lag" period was evident in all instances, but this was so pronounced with the milk spoilage organisms as to warrant the term dormancy. Although it is not shown in the table, this variability of response was not confined to certain organisms, for various tests on a single strain often resulted in very different germination times in the deficient mediums. The spores of strains I and II of the milk organism were particularly susceptible to the stimulating influence of the tomato and yeast mediums. *B. cereus* was the least exacting in its nutritional requirements. The spores of *B. cereus*

TABLE 1

Influence of medium on the germination of washed aerobic spores

ORGANISM	MACROSCOPIC GROWTH, INCUBATION AT 37°C.						
	Sugar-free broth	2 per cent peptone solution	Plain broth	Glucose broth	Tomato extract broth	Yeast extract broth	Synthetic medium
Milk strain I:							
Growth 12 hours	0	0	0	++	++++	++++	0
Delayed growth	+ 17 days	+ 30 days	+ 5 days				0 30 days
Milk strain II:							
Growth 12 hours	0	0	0	++	++++	++++	0
Delayed growth	+ 16 days	+ 3 days	++ 3 days				0 30 days
B. cereus:							
Growth 12 hours	0	0	+++	+++	++++	++++	0
Delayed growth	+ 18 hours	+ 18 hours					0 30 days
B. vulgatus (Ford):							
Growth 12 hours	0	0	++	+++	++++	++++	0
Delayed growth	+ 1 day	+ 1 day					0 30 days
B. subtilis:							
Growth 12 hours	0	+	+++	+++	++++	++++	0
Delayed growth	+ 1 day						++ 2 days

+, light growth; ++, medium growth; +++, heavy growth; ++++, extremely heavy or luxuriant growth.

apparently germinate as rapidly in plain broth as in tomato-extract medium, as determined by the interval plate counting method (see table 2), although in the macroscopic tests growth in the tomato medium seemed to be more luxuriant than in plain broth.

2. The influence of temperature on the germination of washed spores

It is commonly supposed that non-thermophilic, aerobic spore-forming bacteria, except *B. anthracis*, have, as their optimum for

TABLE 2

Influence of medium on the germination of washed aerobic spores

ORGANISM	MEDIUM	GERMINATION INDICATED BY INTERVAL PLATE					
		0 hours	1 hour	3 hours	5 hours	7 hours	9 hours
Milk strain I	Plain broth	9,600	9,000	6,800	9,400	9,100	9,000
	Tomato broth	6,200	12,500	13,800	32,000	51,000	*
Milk strain II	Plain broth	700		600		800	
	Tomato broth	1,100		2,900		9,000	
B. anthracis	Plain broth	13,000	18,000	56,000	*	*	*
	Tomato broth	16,000	26,000	96,000	*	*	*
B. subtilis	Plain broth	36,000	30,000	32,000	42,000	120,000	*
	Tomato broth	38,000	62,000	86,000	166,000	*	*
B. cereus	Plain broth	9,000	12,000	36,000	*	*	*
	Tomato broth	7,200	10,000	32,000	*	*	*
B. vulgatus	Plain broth	1,400	1,700	1,800	6,300	19,600	*
	Tomato broth	1,600	1,800	3,300	9,700	*	*

* Countless in the dilutions plated.

growth, temperatures ranging from 24° to 30°C. In table 3 the temperature ranges for the germination of washed spores of several representative species are presented; they show that, in so far as rapidity of germination is concerned, temperatures of 37°C., and in most instances, 48°C., are much more effective than the lower temperatures. Although all but one of the test organisms were capable of germination and growth at 12°C. there was a

TABLE 3

Influence of temperature on the germination of washed spores

INCUBATION TEMPERATURE	TIME OF INCUBATION	GERMINATION OF SPORES (APPEARANCE OF COLONIES)				
		B. anthracis	B. subtilis	B. cereus	B. vulgatus	Milk strain II
°C.						
6	12 hours	0	0	0	0	0
	24 hours	0	0	0	0	0
	60 hours	0	0	0	0	0
	16 days	0	0	0	0	0
12	12 hours	0	0	0	0	0
	24 hours	0	0	0	0	0
	60 hours	+	0	+	0	0
	Delayed		+ 7 days		+ 7 days	0 16 days
16	12 hours	0	0	0	0	0
	24 hours	+	0	+	0	0
	60 hours		+		+	0
	Delayed					+ 10 days
20	12 hours	0	0	0	0	0
	24 hours	+	0	+	0	0
	60 hours		+		+	+
	Delayed					
24	12 hours	+	0	+	0	0
	24 hours		+		+	0
	60 hours					+
	Delayed					
30	12 hours	+	+	+	+	0
	24 hours					+
	60 hours					
	Delayed					
37	12 hours	+*	+	+*	+	+
	24 hours					
	60 hours					
	Delayed					
48	12 hours	0	+*	0	+*	+*
	24 hours	0		0		
	60 hours	0		0		
	Delayed	0		0		
60	12 hours	0	+	0	0	0
	24 hours	0		0	0	0
	60 hours	0		0	0	0

Standard nutrient agar.

* Optimum in time of germination; greater number of colonies appearing.

variable lag at this temperature. With *B. subtilis* and *B. vulgatus* the first visible colonies appeared in seven days, which would indicate an actual delay in germination, rather than simply a slow rate of growth after germination had taken place. Further evidence in this direction was obtained from a numerical determination of the effect of temperature on germination. Plate counts on washed spore suspension inoculum showed that, within the temperature range for germination, the number of colonies appearing became progressively larger as the temperature increased. Thus, at 48°C. the average count was approximately four times as great as the average count at 12°C. and in most instances as that at 16°C., within the maximum incubation time of 16 days. Thus, it seems that the germination of the majority of the spores in a given amount of suspension is indefinitely delayed at the lower temperatures.

The conclusion that low temperature is an environmental factor to which these spores must become adapted before germination can ensue, seems justifiable.

3. The influence of gaseous environment on the germination of washed aerobic spores

In preliminary experiments with washed aerobic spores it was found that the substitution of atmospheres containing variable amounts of oxygen in mixture with carbon dioxide, ethylene and other gases, in place of ordinary air, exerted little or no influence on the germination of the spores.

It is generally supposed that certain minimal amounts of oxygen are required for the germination of aerobic spores, but this seems to hold only for some of the organisms employed in the present work. Meyer (1909) and others have considered oxygen availability as one of the cardinal factors in spore germination. Table 4 gives the results of the effect of vacuum[1] on the germination of several representative species of aerobic organisms on the surface of agar medium. The data reported are based on numerous experiments in which complete anaerobiosis was obtained as

[1] The term "vacuum" is used here in a relative, and not absolute, sense. The same applies to anaerobiosis."

TABLE 4

Gaseous environment. Influence of vacuum and of various concentrations of ethylene on the germination of washed aerobic and anaerobic spores

ORGANISM	VACUUM ONLY			EVACUATED JARS REPLACED WITH ETHYLENE													
				10 per cent ethylene			25 per cent ethylene			50 per cent ethylene			75 per cent ethylene			100 per cent ethylene	
	Hours incubated at 37°C.																
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
*B. anthracis**	0	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. cereus	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. subtilis	0	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. vulgatus (1)	0	0	0	0	+	+	0	+	+	0	+	+	0	+	+	0	+	+
B. vulgatus (2)	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Milk strain I	0	0	0	0	+	+	0	+	+	0	0	+	0	+	+	0	0	+
Cl. sporogenes	0	±	++	0	++	++	0	++	++	0	++	++	0	++	++	0	0	++
Cl. botulinum 38	0	0	0	0	0	+	0	0	+	0	0	+	0	+	+	0	0	+
Cl. botulinum 73	0	0	+	0	+	++	0	+	++	0	0	+	0	+	++	0	0	+
Cl. botulinum 619	0	+	++	+	++	++	+	++	++	0	+	++	+	++	++	0	+	++

* Slightly better growth in ethylene than other organisms.

0, no growth; ±, scant growth; +, definite but not optimal growth; ++, heavy or maximum growth.

shown by the manometric readings and by the sensitive methylene blue indicator used in all of the vacuum jars. In some of the experiments in which these same results were obtained, oxygen absorption was resorted to in the vacuum jars, supplementary to evacuation. For this purpose sodium hydrosulphite in alkaline solution was placed in the bottom of the jars. A Cenco Hyvac pump was used in this work. According to the results obtained, no measurable minimum oxygen requirement can be established for the germination of spores of the strains of *B. anthracis*, *B. subtilis* and *B. vulgatus* (I) used. It is significant that *Cl. sporogenes* and various strains of *Cl. botulinum* developed on the surface of the standard nutrient agar under the same conditions (in the same or separate vacuum jars), and in approximately the same time as did the three so-called aerobic species mentioned above.

The influence of ethylene gas on the germination of aerobic and anaerobic spores. Gaseous environments in which unsaturated gases like acetylene and ethylene are incorporated have not been studied in connection with the germination of bacterial spores, in so far as the writers have been able to determine. Several considerations, particularly the striking physiological effects exerted by ethylene on the cells of growing plants (Harvey, 1915), the stimulation of the germination of seeds and the sprouting of dormant plant organs by this gas (Rosa, 1925), and the acceleration of the ripening of fruits and vegetables by ethylene, probably due to stimulation of the enzymic function and other vital cell activities, prompted the following investigation of the effect of various concentrations of ethylene gas on spores. Since this gas is stimulative to various biological processes, an analogous acceleration of spore germination was considered possible.

Since no stimulation was detectable in the preliminary experiments, in which broth and agar mediums inoculated with washed aerobic spores were incubated in bell jars containing atmospheres made up of different mixtures of ethylene with oxygen or air, experiments were undertaken to determine the effect of ethylene on the germination of aerobic and anaerobic spores *in vacuo*. It will be seen from table 4 that, compared with vacuum only, ethylene does apparently stimulate the germination of both aerobic

and anaerobic spores under identical conditions. This suggests that the effect involves a direct stimulation of vital forces, perhaps of the enzymic function of the spores. It is noteworthy, also, that even in the highest concentration attainable ethylene is non-toxic to the sporulating bacteria. Although maximum growth of the aerobic organisms, following germination from the spore, does not take place *in vacuo* or in any concentration of ethylene displacing the vacuum, this effect is not due to toxicity of the gas, since growth takes place about equally well in all concentrations, and since uniform, maximum growth ensues when agar slant cultures are removed from the vacuum jars and incubated under atmospheric conditions, though the exposure to ethylene has been continued for ten days or more.

4. Nutritional factors influencing the dormancy of heated washed spores

Since the germination of washed unheated spores of the milk organism was markedly stimulated by the nutritional factors supplied in tomato or yeast-extract mediums, as compared with standard nutrient broth and other mediums, parallel heating experiments were conducted to determine whether or not these same media exert the same favorable influence on spores that have been heated, keeping in mind the fact that the spores of the milk organism exhibited pronounced and variable dormancy in all previous instances when heated in water and subcultured in standard nutrient broth. In these experiments a single tube series of spore suspensions, all from the same stock, was heated in buffered distilled water at 105°C. At the end of each time interval a single tube was removed trom the oil bath, and equal portions of its contents were subcultured in the different mediums. The results are shown in table 5.

The striking points in these comparative tests in which equal portions of single heated suspensions were subcultured in different mediums are: (*a*) That the same pronounced "dormancy" or delayed germination occurred in the standard nutrient broth subcultures in all of the individual tests on heated spores; (*b*) that in the tomato and yeast-extract subculture mediums dormancy

and skips were entirely eliminated, growth occurring promptly (within twelve to twenty-four hours) in every tube of the single tube series up to the critical time; and (*c*) that heat shock or injury can play no part in the dormancy of this organism, since spores receiving the maximum heat germinate as promptly as others when a favorable environment is supplied in the subculture medium.

TABLE 5

Influence of nutritional factors on the dormancy of spores after heating

TEMPERATURE HEATED	TIME HEATED	MILK STRAIN I (SUBCULTURES AT 37°C.)			MILK STRAIN II (SUBCULTURES AT 37°C.)			
		Growth in 12 to 24 hours		Delayed growth in plain broth	Growth in 12 to 24 hours			Delayed growth in plain broth
		Tomato medium	Plain broth		Tomato medium	Yeast medium	Plain broth	
°C.	minutes							
105	15	+	−	+ 16 days	+	+	−	+ 2 days
105	30	+	−	+ 16 days	+	+	−	+ 2 days
105	40	+	−	+ 22 days	+	+	−	+ 2 days
105	50	+	−	+ 18 days	+	+	−	+ 13 days
105	60	+	−	+ 24 days	+	+	−	+ 30 days
105	70	+	−	+ 22 days	+	+	−	+ 10 days
105	80	+	−	+ 22 days	+	+	−	+ 16 days
105	90	+	−	+ 30 days	+	+	−	− 50 days
105	100	+	−	− 50 days	+	+	−	− 50 days
105	115	+	−	− 50 days	+	+	−	− 50 days
105	130	+	−	− 50 days	−	−	−	− 50 days
105	145	−	−	− 50 days	−	−	−	− 50 days
105	160	−	−	− 50 days	−	−	−	− 50 days
105	180	−	−	− 50 days	−	−	−	− 50 days
105	200	−	−	− 50 days	−	−	−	− 50 days

5. Factors contributing to or determining the dormancy of washed unheated bacterial spores

The method employed in this work for the determination of dormancy follows, with certain important modifications, the procedures worked out by G. S. Burke (1923) and by Victor Burke and his associates (1925) for the study of "normal dormancy" in spores of *Cl. botulinum* and in common aerobic spores.

Technic. Approximately single spores of the various organisms studied were inoculated into deep tubes of different mediums containing 0.5 per cent agar. To this end, washed spore suspensions were diluted and subjected to the plate count method to determine the amount of inoculum necessary for the desired distribution of spores. After selecting a definite dilution, trial tubes of soft agar and of liquid mediums were inoculated with the determined amounts, incubated for twenty-four hours and examined before the regular series of tubes were inoculated. During this interval the dilutions were held in the refrigerator. In inoculating parallel sets of tubes with the same suspension in order to determine the relative stimulation of the germination of the single spores in different mediums, the tubes were seeded alternately from the same pipette. Furthermore, the various mediums were always prepared at the same time and, as far as possible, from the same materials. Other conditions, such as temperature of the medium when inoculated, incubation temperatures, the time of sealing the tubes, etc., were kept constant in parallel tests. The theoretical total of spores in the amount of inoculum used for all of the tubes in a given test was calculated from an average of multiple plate counts of the dilution used. Tubes showing no growth in twenty-four hours were sealed with paraffin and observed daily during the first ten days and at less frequent intervals thereafter.

The results of these experiments on the dormancy of individual washed spores of common aerobic organisms are presented in tables 6 and 7, but a summary of these results seems to be desirable here in order to emphasize certain of the observations.

B. subtilis. It will be seen in table 6 that in an extensive series of tubes distributed through three different mediums there is no evidence of dormancy or delayed germination of the spores of *B. subtilis* within the maximum incubation period of fifty days; all of the tubes showing growth did so within twenty-four hours. The yeast-extract and glucose infusion agars have apparently no advantage over the plain agar for this organism, although the percentage of the theoretical total spores developing in the yeast medium is considerably higher than in the plain or glucose infusion agars. Since no further spores develop in any of the mediums between the first and fiftieth days, the variation in percentages is assumed to be due to an experimental error in the distribution of the spores. This is in accord with the observations

TABLE 6

Results of experiments on the dormancy of individual washed spores

Incubation time	Plain 0.5 per cent agar; 213 tubes inoculated			Yeast-extract 0.5 per cent agar; 110 tubes inoculated			Glucose-infusion 0.5 per cent agar; 40 tubes inoculated		
	Theoretical total spores in inoculum: 213			Theoretical total spores in inoculum: 110			Theoretical total spores in inoculum: 40		
	Tubes positive	Total colonies all tubes	Per cent of theoretical total spores	Tubes positive	Total colonies all tubes	Per cent of theoretical total spores	Tubes positive	Total colonies all tubes	Per cent of theoretical total spores
A. B. subtilis									
days									
1	143	206	96.7	70	115	104.5	26	34	85.0
2									
3									
50									
	143	206	96.7	70	115	104.5	26	34	85.0
B. B. megatherium	50 tubes inoculated			50 tubes inoculated			50 tubes inoculated		
	Theoretical total spores in inoculum: 38			Theoretical total spores in inoculum: 38			Theoretical total spores in inoculum: 38		
1	23	27	71.0	26	36	94.7	1	1	2.6
2	4	5	13.2	1	1	2.6			
3	1	1	2.6						
4		1	2.6						
5		2	5.3						
11	1	1	2.6						
45									
	29	37	97.3	27	37	97.3	1	1	2.6
C. B. cereus	25 tubes inoculated			25 tubes inoculated					
	Theoretical total spores in inoculum: 22			Theoretical total spores in inoculum: 22					
1	14	20	90.9	14	16	72.7			
2									
45									
	14	20	90.9	14	16	72.7			
D. B. vulgatus	50 tubes inoculated			50 tubes inoculated					
	Theoretical total spores in inoculum: 28			Theoretical total spores in inoculum: 28					
1	22	24	86.0	18	24	86.0			
2				2	2	7.1			
45									
	22	24	86.0	20	26	93.1			

made previously that this strain of *B. subtilis* is not at all exacting in its nutritional requirements, growth occurring about as well in plain broth as in glucose broth or in any other special medium. The fact that this organism was the only one of those tested which developed from the spore in the synthetic medium further confirms this assertion.

B. megatherium. In the experiments with *B. megatherium* an inexplicable idiosyncrasy was displayed by the spores. Within the maximum period of incubation only one spore out of a theoretical 38 germinated in the glucose infusion 0.5 per cent agar, although the three mediums indicated in table 6, B, were inoculated alternately from the same pipette with the same amount of inoculum. It is apparent from the table that this effort to insure even distribution of the spores was successful in so far as the first two mediums are concerned. The glucose infusion medium employed was part of the same batch which was used in the experiment with *B. subtilis* and, as has been shown, the spores of *B. subtilis* germinated as well in this as in the other mediums. Since conditions were kept constant in all other details of this parallel test it must be assumed that these results are due to subtle peculiarities of these spores in their nutritional requirements for germination. It will be noted in this experiment also that the period of dormancy extends to the eleventh day in the plain agar, with 26.3 per cent of the total spores developing after 24 hours. In the yeast-extract medium dormancy is practically eliminated, extending only to the second day, with one spore or 2.6 per cent. This further indicates special requirements for germination of the spores of this organism.

B. cereus and B. vulgatus. Although no very definite conclusions can be drawn from the limited number of tubes inoculated with single spores of these organisms, as shown in table 6, C and D, the indication is that they do not exhibit particular tendencies toward dormancy under the environmental conditions supplied.

Milk strain I. The most marked peculiarities in requirements for germination observed in these experiments, and the most prolonged periods of dormancy, were exhibited by the spores of strain I of the milk organism. As was expected, on the basis of

the earlier experiences with this organism and the dormancy displayed by it in plain broth subcultures after heating, the indi-

TABLE 7

Results of experiments on the dormancy of individual washed spores. Milk Strain I

INCUBATION TIME	PLAIN 0.5 PER CENT AGAR; 100 TUBES INOCULATED			YEAST-EXTRACT 0.5 PER CENT AGAR; 100 TUBES INOCULATED			GLUCOSE-INFUSION 0.5 PER CENT AGAR; 45 TUBES INOCULATED		
	Theoretical total spores in inoculum: 100			Theoretical total spores in inoculum: 100			Theoretical total spores in inoculum: 45		
	Tubes positive	Total colonies all tubes	Per cent of theoretical total spores	Tubes positive	Total colonies all tubes	Per cent of theoretical total spores	Tubes positive	Total colonies all tubes	Per cent of theoretical total spores
days									
1	0			66	78	78.0	8	8	17.7
2	0			9	9	9.0	4	4	8.8
3	0			1*	1*	1.0	1	1	2.2
4	0						1	1	2.2
5	0						2	2	4.4
6	0						1	1	2.2
8	0			1*	1*	1.0	2	2	4.4
9	0			2	2	2.0	1	1	2.2
10	0			2	2	2.0	1	1	2.2
12	0						2	2	4.4
14	0			2	2	2.0	2	2	4.4
16	0			3	3	3.0	3	3	6.6
17	0			2	2	2.0	2	2	4.4
19	0			2	2	2.0	1	1	2.2
21	1	1	1.0				1	1	2.2
22	1	1	1.0						
24	2	2	2.0						
26	1	1	1.0				2	2	4.4
30	2	2	2.0						
35	5	5	5.0				1	1	2.2
40	4	4	4.0				1	1	2.2
45	11	11	11.0						
50	6	6	6.0						
	33	33	33.0	90	102	102.0	36	36	80.0

Surface growth.

vidual washed unheated spores showed pronounced dormancy in the plain deep 0.5 per cent agar (table 7). In glucose infusion

agar variable periods of dormancy were encountered; yet, unlike the action in plain agar, germination began on the first day, showing that the spores were supplied with better, though not optimal, environmental conditions. Carrying this observation further, it is seen that conditions still more favorable, and yet not optimal, are supplied in the yeast-extract agar, as evidenced by the fact that 78 per cent of the theoretical total number of spores developed within 24 hours in this medium, whereas only 17.7 per cent of the spores in glucose infusion agar showed growth in the same time.

The fact that 78 per cent of the individual spores of this organism developed promptly in the yeast-extract agar, while there was a prolonged quiescence in the plain agar medium, offers an explanation of the elimination of dormancy in heat resistance tests on this organism by substituting yeast-extract medium for plain broth in the subcultures. The objection is of course raised that the final criterion as to whether or not an organism has tendencies toward dormancy, according to definition, is the prolonged quiescence of a few spores out of a transplant, and that since only 78 per cent of the total single spores showed prompt germination, this organism has inherent tendencies toward dormancy. This may or may not be the case, and in the discussion that is to follow several factors will be considered which should enable us to form at least an hypothetical conception of the factors which determine dormancy.

It may be said here, however, that the graduated results ob tained with this organism in three different mediums—pronounced dormancy in plain agar, less marked dormancy in glucose infusion agar and still less in yeast-extract medium,—suggest that the dormancy is simply an index of the degree of unfavorableness of the environment or medium rather than a normal or inherent quality of the spores.

6. Discussion

The present work has shown that bacterial spores may reveal marked variability in their response to environmental influences. It seems justifiable to assume that spores in the process of germination are active metabolizing bodies, having exacting nutri-

tional and other requirements for germination. In the absence of particularly favorable conditions, spores undergo a period of adaptation just as growing vegetative cells do under changes of environment (Lag phase). When, however, unfavorable environmental conditions are encountered by spores, the lag period is usually so extended that it acquires the aspect of what is termed dormancy.

Of the various nutritional factors influencing spore germination, and therefore spore dormancy, the availability of nitrogen is perhaps of outstanding importance. From the work of Sperry and Rettger (1915), Rettger, Berman and Sturges (1916) and Berman and Rettger (1918) it is evident that the nutritional value of a culture medium is largely dependent upon the nature of the nitrogenous material present, and that amino acids and other substances which readily give up their nitrogen are of particular significance. Although their work was not directly concerned with spore germination, it is readily applicable to this process on the basis of the foregoing assumption that spores are not inert, but active bodies having the same or more exacting requirements for their metabolism than do vegetative cells. Stickel and Meyer (1918) suggested the general use of peptic or tryptic digestion products of protein material in bacteriological work, particularly in the cultivation of the sporulating anaerobes, due to the high amino acid content and nitrogen availability of such mediums as compared with the usual standard mediums. Among the numerous investigators suggesting the use of various forms of yeast autolysate or extract mediums, either because of their growth-stimulating properties or as economical substitutes for meat extracts, are Kressler (1918), Kligler (1919), Eberson (1920) and Ayers and Rupp (1920). Thjötta (1921) and Thjötta and Avery (1921) made important contributions to the subject of bacterial stimulation by growth accessory substances in certain culture mediums. In the latter communication they reported marked stimulation of bacterial growth by small amounts of extracts of yeast cells, and of certain vegetables such as green peas, string beans and tomatoes. Wagner, Dozier and Meyer (1924) emphasized the high "biologic value" of mediums contain-

ing beef heart infusion, and the superiority of these mediums over others in the original isolation of the anaerobic bacteria from spores.

That incubation temperature and heat stimuli may play important rôles in bacterial spore germination is evidenced by the results of the experiments on the temperature ranges for germination reported herein, and by the work of previous investigators. Weyer and Rettger (1927) showed that marked stimulation of the germination and subsequent vital activity of spores of *Cl. acetobutylicum* followed a brief exposure of the spores to boiling temperature. Also, Allen (1923) in his work with spores of *B. subtilis* showed that more rapid germination and multiplication took place after heat treatment or shock at 145°F. for 30 minutes than in the unheated material.

Ludwig (1918) contributed the only direct work on the effect of ethylene gas on bacteria that has come to the attention of the writers. His work was concerned with the influence of illuminating gas and its constituents on bacteria and fungi, particularly varieties pathogenic for plants. He found that the development of bacteria in the presence of ethylene was not materially checked until high concentrations were reached. In spite of the various stimulative properties of ethylene, it has been reported as havng extreme toxicity for certain plants and plant organs. Crocker and Knight (1908) reported on the toxic effect of ethylene upon flowering carnations. Knight and Crocker (1913) noted extreme toxicity for sweet pea epicotyls. Harvey (1915), and Harvey and Rose (1915) have shown that the extreme toxicity of ethylene holds for numerous different species of plants. Ludwig, in the work just cited, was seeking an analogous toxicity for bacteria and fungi that are pathogenic for plants.

Other than to suggest that gaseous environment may enter as one of the factors determining germination and hence dormancy, and especially that the unsaturated hydrocarbon gases such as ethylene may act as stimulants of germination, the present work on gases is not sufficiently complete to be conclusive. If, however, the writers' hypothesis relative to the facilitation of spore germination by means of pre-germination enzymic function be

taken into consideration, an hypothetical explanation of ethylene stimulation may be suggested; namely, that ethylene accelerates this enzymic or other metabolic function of the spores. The recent work of Nord and Franke (1928) tends to support this explanation. Their paper dealt with the protective and stimulative effect of ethylene on various enzymes, either in solutions (cell extracts) or in living cells (zymase of yeast) or in plant tissues (catalase of tobacco leaves).

In the experiments on the dormancy of heated spores of the milk organism it was conclusively shown that the dormancy exhibited by these spores in standard nutrient broth subcultures could not have been "normal" dormancy, or due to factors of "heat inhibition" or "heat shock," to which previous investigators have usually attributed prolonged dormancy after heating. Spores of single heated suspensions showed prompt germination when supplied with adequate nutritional stimuli in the subculture medium, that is by simple substitution of tomato or yeast-extract medium for standard nutrient broth. This observation seems significant, since standard nutrient broth is commonly used as the subculture medium in heat resistance determinations on common aerobic spores and in sterility tests of various kinds.

In the experiments on the dormancy of individual washed spores of aerobic bacilli there is further evidence that the dormancy of unheated spores, usually called "normal dormancy," is in reality a function of the environment or an index to the degree of unfavorableness of the environment of the spores, since the extent of dormancy varied markedly according to the nutritional stimuli supplied in the medium. It was observed that *B. subtilis*, *B. cereus* and *B. vulgatus* did not show any tendency toward so-called "normal" dormancy when single washed spores were inoculated into plain, glucose infusion or yeast-extract soft agar mediums. This observation is at variance with the findings of Burke *et al.* (1925), who reported that unheated spores of *B. subtilis* remain dormant for as long as 39 days in deep agar culture. Differences in the strains of *B. subtilis* used, or in the technic, might well account for this discrepancy in results. In determining dormancy, Burke inoculated approximately single, unwashed,

unheated spores, or rather growth from an agar surface of sufficient age to insure the presence of spores, into standard 1.5 per cent deep nutrient agar containing 0.5 per cent glucose. With *B. megatherium* and strain I of the milk organism, however, there were marked and variable periods of dormancy in the plain soft (0.5 per cent) agar medium employed in the present work, as shown in tables 6 B and 7.

In discussing the factors contributing to, or determining, dormancy under experimental conditions, it will be perhaps more convenient to present the additional observations and comments under headings denoting the principal factors thought to be of significance:

a. Consistency of the medium. Preliminary tests with the *B. subtilis* strain employed here showed that deep tubes of standard agar, such as those used by Burke, do not supply "conditions favorable for growth," as evidenced by the fact that development is slow and the colonies extremely small. In deep tubes of the soft agar medium, on the other hand, colonies developed to a maximum size of 6 mm. in diameter in the same or shorter periods of time. While it was the writers' belief that deep agar culture in any form is not favorable for the germination of aerobic spores, the convenience of the method and the fact that *B. subtilis* grew well in the soft agar led to its adoption for want of a better method, particularly a surface cultivation method.

In the course of the work it was observed that cells developing from the inoculated spores were capable of motility in the 0.5 per cent agar. It is obvious that such a condition would permit the cells of the developing colonies to utilize the nutrient material present in a considerable area surrounding the original loci of development. Furthermore, it is conceivable that, although spores imprisoned in 1.5 per cent solid agar may germinate, the subsequent growth may be checked or even inhibited due to the accumulation of metabolic products or to a mere mechanical obstruction to the free movement of the cells. Mechanical and physical factors such as density of the medium, surface tension, osmotic pressure and oxygen penetration, then, must be taken into account as serious objections to the deep agar culture method

of determining dormancy, particularly if solid agar of standard composition be used. In substantiation of this point it should be said that spores of strain I of the milk organism, for example, develop promptly on the surface of plain agar while, as seen in the results in table 7, the first sub-surface colonies to appear in the deep 0.5 per cent plain agar did so only after 21 days.

b. Oxygen tension. An observation was made that suggests a further explanation of the occurrence of dormancy under conditions of deep agar culture; namely, that with every organism tested the cells invariably moved through the soft agar from the original loci of development toward the surface of the medium. In all cases, however, initial colonies developed at the points of germination of the spores. These were usually fluffy or of "puff ball" appearance and on continued incubation tended to diffuse through the medium. It was frequently observed that from a more or less dense initial locus the colony would spread out radially in the direction of the surface of the medium but never toward the bottom of the tube. It was also noted in a few instances in which there were several colonies distributed throughout a single tube that the colonies were increasingly larger as the surface of the medium was approached, those nearest the top being many times the size of those at the bottom of the tube. The natural conclusion from these observations is that environmental conditions in the deep agar are unfavorable to aerobic bacteria, due to decreased oxygen accessibility. This factor alone may determine, or at least contribute to, dormancy of spores, although it was apparently not operative in the case of *B. subtilis*, which showed no delayed germination. It should be said, however, that the strain of *B. subtilis* used was capable of germination and growth under essentially anaerobic conditions.

c. Enzymic function. In a previous communication (1930) the writers have shown that the milk organism produces an active substance that is capable of bringing about marked changes in milk, probably a caseolytic enzyme, and the hypothesis was advanced that this enzyme may aid in, and perhaps account for, the prompt germination of spores of this organism in milk, even after severe heating. On the basis of the assumption that spore

germination may be facilitated by enzyme secretion, another factor contributing to the dormancy of aerobic spores in deep agar culture may be cited. Effront (1917) has shown that *B. mesentericus*, when cultivated on large surfaces, produces twenty times as much protease as when grown below the surface. This suggests of course that the enzymic function of this organism is dependent upon oxygen accessibility. Since protein metabolism is probably of great importance during the process of germination, the function of the proteases alone may determine the dormancy of spores in some instances.

d. Single spore inoculum. There has been considerable controversy on the question of the mutual accelerative action of organisms in freshly seeded cultures. It is the view of numerous workers that such an action is accountable for prompt growth in a new environment, and that the inevitable result of dilution and isolation of the cells is lag. Sherman and Albus (1924), however, in answer to the question "Is there an allelocatalytic effect in the growth of bacterial cultures?" take the stand that the rate of growth of a culture is constant regardless of the size of the inoculum.

Another view is that growth ensues more rapidly in cultures started from large inoculums due to the fact that they contain assortments of individuals, some of which are more vigorous or capable of adaptation to new environmental conditions than others. That this view does not necessarily apply to spore inoculums is shown in the present experiments with single spores of *B. subtilis*, the individual spores of which developed as rapidly as did larger spore seedings. The work of Barber (1920) adds weight to this contention; in using the single cell isolation technic, he found that spores of *B. subtilis* gave 100 per cent positive cultures, and that the spores developed promptly even when transferred to glucose broth medium covered with oil or vaseline to provide anaerobic conditions. It may be concluded, then, that the isolation of the individual spores is unimportant as a factor contributing to dormancy, at least with the organisms investigated here.

e. Nutrient value of the culture medium. The effect on germination of the nutrient value of the culture medium has been already

discussed to some extent. With washed unheated spores of a number of organisms it has been shown that very definite retardation or acceleration of germination may occur, depending upon the kind of nutrient material that is present in the medium. The experiments on the dormancy of single washed unheated spores further emphasize the importance of adequate nutritional stimuli in spore germination. The fact that with some organisms the extent of dormancy is graduated in different mediums in which the spores of other organisms develop rapidly and equally well indicates that there are definite nutritional requirements for different organisms, and that the lack of these readily causes, or contributes to, dormancy.

SUMMARY

Bacterial spore dormancy and germination have been investigated in relation to environmental factors.

Because of the marked variability of germination, depending upon the stimuli supplied in the environment, the deduction is made that bacterial spores in the process of germination are vitally active bodies having requirements for metabolic function which are the same as or more exacting and specific than those of the vegetative cells.

Experimental evidence is presented to show that the dormancy of aerobic bacterial spores is largely, if not entirely, determined by conditions in the environment of the spores, and that these factors must be taken into consideration, perhaps specifically for each species, before so-called "inherent" or "normal" dormancy of bacterial spores can be established.

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A STUDY OF BACILLUS NECROPHORUS OBTAINED FROM COWS

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Bacillus necrophorus is an important organism in animal disease since it produces severe septic processes in a number of domestic animals. The organism is widely distributed and is responsible for considerable losses. It may invade almost any tissue and is associated with various necrotic foci, as in calf diphtheria, necrotic ulcers of the intestine in hog cholera, foot-rot of sheep and cattle, grease heel or necrotic scratches of horses, lip and leg ulceration of sheep, gangrenous dermatitis of horses, metastatic necrosis of liver and lungs of cattle and swine, necrotic stomatitis of calves, lambs and pigs.

A few human cases of infection with a similar organism have been reported. These are referred to by Harris and Brown (1927) who isolated from cases of puerperal infection in women cultures closely resembling *B. necrophorus* and designated by them *Act. pseudo-necrophorus*.

Because of the wide distribution of necrophorus bacilli and the fact that they produce disease in such a variety of animals it seemed worth while to find out as much as possible about strains from different sources. This work has been started with a group of 10 cultures isolated from cows, studies of which are reported in this paper. They include 7 strains isolated from liver abscesses, 1 from a spleen abscess, 1 from a lung, and the last from an abscess in the omentum.

Isolations were made in two ways, either directly through cultures or by passage through a rabbit. If a stained preparation of the original material showed a predominance of necrophorus

bacilli, then the isolation was made directly through cultures. Since the organism grows well on ordinary media plus a little serum, to isolate a pure culture a suspension of the original material in bouillon was inoculated into serum agar shake cultures. Colonies appeared usually in two days. The column of agar was then forced out of the tube into a sterile petri dish by inserting a capillary pipette to the bottom of the tube and exerting pressure on the bulb. Individual colonies could then be picked from the agar and inoculated into serum bouillon for further study.

If the original material showed other bacteria present in any considerable numbers, then a suspension of this material in bouillon was inoculated subcutaneously into a rabbit. Usually the contaminating bacteria disappeared or decreased in numbers while *B. necrophorus* multiplied and produced an infection. From the necrotic tissue or serous fluid or often from the heart blood of the rabbit pure cultures were obtained. However, to establish a pure strain the rabbit material was inoculated into serum agar shake cultures and separate colonies picked from these tubes as described. In the study of these pure cultures anaerobic conditions were maintained by using a vaseline seal on the tubes of media.

The experimental work with these strains consists of (*a*) morphological studies including tests for the existence of spores by staining methods and by testing the resistance of cultures to heat, drying and exposure to air; (*b*) cultural reactions in various media and fermentation results; (*c*) certain serological reactions including agglutination and agglutinin absorption studies; and finally (4) some observations on the pathogenicity of the cultures.

MORPHOLOGY

Morphologically the present series of bovine strains agree in general with the descriptions of *B. necrophorus* in the literature (see bibliography). *B. necrophorus* is a plemorphic organism varying from coccoid forms to filaments 100μ long. All the present strains show these variable forms. One strain differs somewhat from the others by the appearance of the short rod forms sometimes joined together in chains. Young cultures

show chiefly long rods and filaments while older cultures contain mostly shorter rod forms of varying length. The rods are non-motile and negative to Gram's stain. Some elements are evenly stained but often distinctly beaded forms are seen. Sometimes a rod or filament shows a swollen or thickened area appearing either as a short round swelling near the center of the rod or as an elongated thickening toward one end. Beaded forms are characteristic and are clearly shown in preparations stained with carbol fuchsin. Such filaments show clear or faintly stained spots alternating with deeply stained portions. The unstained areas and those which stain may be of equal size although variation in size is not uncommon. Mohler and Morse (1904) have described in detail these various types of beaded forms.

The general appearance of the clear, unstained spots in the rods suggests spores. The question of spore formation is important since this organism is a strict anaerobe and the presence of spores would readily explain its survival outside the body for long periods and thus help to explain its frequency in animal infections. However, all descriptions in the literature state that *B. necrophorus* cultures contain no spores. Most authors mention the occurrence of beaded forms as characteristic for this organism but do not connect this appearance with spores. Mohler and Morse, however, mention that the unstained spaces in the filaments were first thought to be spores but that spore staining methods did not change their appearance.

In the present study the unstained areas, although they suggested spores in general appearance, were considered to differ from true spores on account of the variation in their size and arrangement in the rods or filaments. It was also found that they failed to stain by methods employed for staining spores. Another point indicating lack of agreement between spores and the unstained spaces in the filaments is the fact that the rods of the older cultures, where one would expect to find spores, show fewer beaded forms than the filaments of the young cultures. Mohler and Morse also stated that the longer rods and threads particularly exhibited the beaded appearance.

In order to determine the ability of the unstained spaces in the

filaments to behave as spores, cultures were tested for resistance to exposure to air, drying and heat. The first experiments were made to test the effect of exposure to air at room temperature. Two strains were tested at the same time, and both a three days' and a two weeks' old culture were used. The serum bouillon cultures were poured into petri dishes so as to form thin layers. Subcultures into serum bouillon were made at the start and at various intervals. The effect of this exposure to air is given in table 1.

Later, another series of tests was made with older cultures, since if spores are formed one would expect to find them in such

TABLE 1

Resistance of B. necrophorus cultures to exposure to air

Cultures poured into petri dishes in thin layers and kept at room temperature.

TIME OF EXPOSURE	3 DAYS' SERUM BOUILLON CULTURE OF STRAINS		2 WEEKS' SERUM BOUILLON CULTURE OF STRAINS	
	D	E	D	E
Control (unexposed)	+* (24 hours)	+ (24 hours)	+ (2 days)	+ (2 days)
hours				
4	+ (24 hours)	+ (2 days)	+ (3 days)	−
8	+ (5 days)	+ (4 days)	+ (5 days)	−
11	+ (5 days)	+ (6 days)		
15			−	−
18			−	−
24	−	+ (5 days)	−	−
28	−	−	−	−

* + = growth; − = no growth.

cultures. The results of the test with 9 different strains of three weeks' old serum bouillon cultures are given in table 2.

The results are variable. For instance, no growth occurred in subcultures from Strain D after two hours' exposure to air, from H after four hours, from I after five hours, or from A, C, and E after seven hours. Strains B, F, and G still developed growth after seven hours' exposure to air but only after prolonged periods of incubation of six to nine days; evidently a good many of the original forms had been killed. A third test with a twenty-four

TABLE 2

Resistance of B. necrophorus cultures to exposure to air

Cultures poured into petri dishes in thin layers and kept at room temperature

TIME OF EXPOSURE	3 WEEKS' OLD SERUM BOUILLON CULTURES OF STRAINS								
	A	B	C	D	E	F	G	H	I
Control (unexposed)	+ (2 days)	+ 2 (days)	+ (2 days)	+ (1day)	+ (3 days)	+ (3 days)	+ (2 days)	+ (3 days)	+ (1 day)
hrs.									
1	+ (3 days)	+ (3 days)	+ (3 days)	+ (5 days)	+ (3 days)	+ (5 days)	+ (3 days)	+ (3 days)	+ (1 day)
2	+ (9 days)	−	+ (14 days)	−	+ (12 days)	+ (11 days)	+ (5 days)	+ (5 days)	+ (4 days)
3	−	−	−	−	−	+ (5 days)	+ (9 days)	+ (6 days)	−
4	−	−	−	−	+ (11 days)	+ (5 days)	+ (5 days)	−	+ (6 days)
5	+ (6 days)	+ (6 days)	+	−	+ (6 days)	+ (5 days)	+ (14 days)	−	−
7	−	+ (6 days)	−	−	−	+ (6 days)	+ (9 days)	−	−

TABLE 3

Resistance of B. necrophorus cultures to drying

Two to three drops culture in bottom of sterile tube, left to dry at room temperature. Tested for resistance by adding fresh serum bouillon, sealing with vaseline and incubating.

TIME OF DRYING	24-HOUR SERUM BOUILLON CULTURE OF STRAINS				6-DAY SERUM BOUILLON CULTURE OF STRAINS		
	A	B	C	D	A	B	C
Control (not dried)	+ (24 hours)	+ (24 hours)	+ (24 hours)	+ (24 hours)	+ (24 hours)	+ (24 hours)	+ (24 hours)
days							
1	−	+ (3 days)	+ (5 days)	+ (3 days)	−	−	−
2	−	+ (3 days)	−	−	−	−	−
4	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−

hour growth of 4 strains (A, B, C, D) showed in all cases resistance to exposure to air for 11 hours, but, the longer the culture was exposed to air, the later growth appeared in the subcultures. The controls inoculated with unexposed culture and tubes from the two hours' exposure developed growth in one to two days, but after eight and eleven hours' exposure to air growth in subcultures did not appear until after six to seven days. Comparison between young and older cultures shows in every case that the older culture was apparently less resistant to exposure to air than younger ones. It is clear, that the organism does not behave as a true spore-bearer, and it is also apparent that air has a distinctly toxic effect.

The effect of drying at room temperature was next considered. The details are given in the second group of experiments. Both twenty-four-hour and six-day serum bouillon cultures were employed for the tests. The results are given in table 3.

The strains showed some variation in degree of resistance. Among the twenty-four-hour cultures, A showed no resistance to drying, while B, C, and D survived the first day. C and D were killed the second day while B remained alive, but this strain gave no growth in tests on the fourth and the fifth day. The six-day cultures did not resist even one day of drying. Again, the older cultures showed less resistance than the younger ones. A twenty-four-hour growth of these strains was also mixed with sand and spread out in petri dishes to dry. The control tubes inoculated with freshly mixed culture and sand gave positive growth, but after one and two days of drying a generous inoculation of the sand into fresh media gave no growth.

Since spores are not readily destroyed by heat it seemed desirable to test the effect of certain temperatures on cultures which had the characteristic beaded appearance. The third experiment deals with the effect of heat. The tests were made in two ways. First a serum bouillon culture under a vaseline seal was heated in a water bath at 60°C. and at intervals culture was withdrawn and inoculated into fresh serum bouillon. Five strains (A, B, C, D, J) were tested. The unheated controls all grew in subcultures. Four cultures grew after exposure at 60°C. for one minute, but

all failed to grow after heating at 60°C. for three minutes. When the test was made by inoculating fresh bouillon tubes, sealing with vaseline, then heating and at intervals withdrawing them and incubating, the results were similar to those obtained by the first method.

This apparent sensitiveness of these cultures to heat, drying and exposure to air indicates that the clear unstained areas seen in the rods and filaments are not true spores. The slight resistance of *B. necrophorus* to exposure to air was noted by Hagan (1924) who showed that exposure to air of a fluid culture in a thin layer caused considerable lag in growth on subculture into cooked meat media, but, if the subinoculations were made into bouillon, growth seldom occurred after thirty minutes' exposure to air. On the other hand, Albrecht (1928) refers to a report by Fr. Meyer who found necrose bacilli resistant to heat and drying. He stated that heating at 95°C. for a short time did not necessarily kill the bacilli and that they would remain virulent when dried for eighteen weeks. Albrecht also mentions a report by Hasenkamp stating that necrophorus bacilli are sensitive to heat and drying. Kelser (1927) found that strains obtained from horses were destroyed immediately at 100°C. and in twenty minutes at 65°C.

CULTURAL CHARACTERS

The cultural studies of the strains show in general a uniform group with slight variations. All strains are strict anaerobes. In serum agar shake cultures they always grow 1 to 2 cm. below the surface. The colonies are round or biconvex, smooth, even and compact. The appearance of the colonies differs from the descriptions of others in the literature. Most observers describe the colonies as woolly or fuzzy. Kelser (1927) compares the colonies in agar shake cultures to small tufts of cotton. In the present work, however, all the strains show smooth, even, compact colonies in agar shake cultures and magnification fails to change their appearance. In serum bouillon all grow chiefly as a sediment, usually of soft particles or masses gradually settling together. In plain bouillon the majority of strains show more clouding and a fine sediment, with the exception of two cultures

TABLE 4

Fermentation reactions

In digest bouillon + 1 per cent of sugar. Original pH 7.2

STRAIN	GLUCOSE		LACTOSE		SUCROSE		MALTOSE		MANNITOL		PLAIN BOUILLON CONTROL	
	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas
		cm.						cm.				
A	5.8	+7.7	6.4	− (bubble)	6.8	− (bubble)	5.9	+6.0	6.6	− (bubble)	7.0	− (bubble)
B	6.0	+3.7	6.6	− (bubble)	6.6	− (bubble)	6.0	+4.2	6.6	− (bubble)	7.0	− (bubble)
C	5.9	+3.5	6.6	− (bubble)	6.6	− (bubble)	5.9	+8.3	6.6	− (bubble)	7.0	− (bubble)
D	6.0	large bubble	6.6	− (bubble)	6.6	− (bubble)	6.0	+3.0	6.6	− (bubble)	7.0	− (bubble)
E	5.8	+10.5	6.6	− (bubble)	6.6	− (bubble)	6.0	+6.7	6.6	− (bubble)	7.0	− (bubble)
F	6.0	+14.5	6.6	− (bubble)	6.8	− (bubble)	6.0	+6.0	6.8	− (bubble)	7.0	− (bubble)
G	5.8	+9.5	6.6	− (bubble)	6.4	− (bubble)	5.9	+8.7	6.4	− (bubble)	6.9	− (bubble)
H	5.8	+9.9	6.4	− (bubble)	6.4	− (bubble)	5.9	+5.2	6.4	− (bubble)	6.9	− (bubble)
I	5.8	+3.5	6.4	− (bubble)	6.6	− (bubble)	5.9	+6.1	6.6	− (bubble)		
J	5.9	+4.0	6.6	− (bubble)	6.6	− (bubble)	5.9	+8.4	6.6	− (bubble)		

(B and I) which always grow chiefly as suspended particles and sediment. These two cultures also agglutinate spontaneously with complete clumping in normal salt solution. The growth in digest bouillon seems slightly heavier than in ordinary veal infusion broth and the addition of glucose increases the growth. All cultures produce indol, hemolyze blood corpuscles, coagulate milk, and do not liquefy gelatin. Two strains (A and B) were inoculated into a series of fermented bouillon tubes of different pH values. The results in these cases indicated that the limiting H-ion concentrations within which growth could occur were pH 6.0 to 8.4. Gas is produced in most media, in serum bouillon, serum agar shake cultures, and to a considerable degree in cooked meat media. Sometimes a bubble of gas is formed in plain infusion bouillon and a large bubble always appears in plain digest bouillon. Two strains which have been tested for H_2O_2 formation when exposed to air gave positive results.

These bovine strains as judged by fermentation reactions in various carbohydrates form a fairly uniform group. The complete seriesof tests were made in digest bouillon to which sufficient carbohydrate had been added to make a 1 per cent solution. A few strains were also tested in fermented bouillon. The fermentation results are given in table 4.

Glucose and maltose were fermented with both acid and gas production with the exception of Strain D which formed very little gas in glucose. The pH in all the other sugar tubes was slightly decreased and no more gas production occurred than that observed when plain bouillon tubes were inoculated. The comparison of digest and fermented bouillon showed practically no difference in the final pH values but there was little gas production in the tubes of fermented bouillon + glucose.

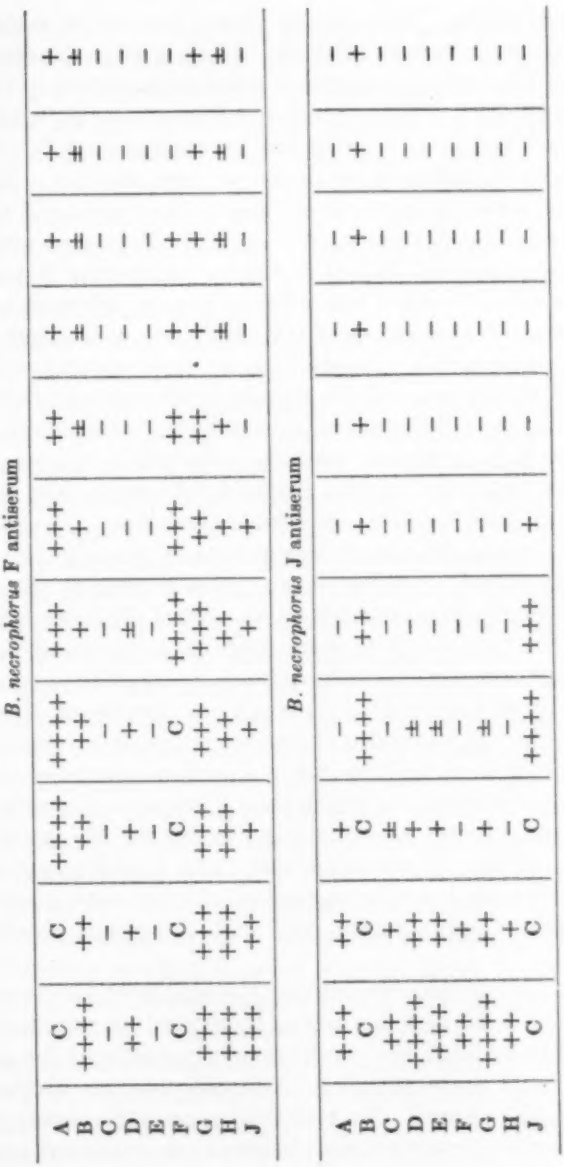
SEROLOGICAL REACTIONS

The group, then, appeared from a morphological and cultural point of view as relatively homogeneous and, as will be shown later, the strains possessed certain pathogenic properties in common. It seemed of interest to determine whether or not the group was a compact one immunologically as judged by common

TABLE 5

Agglutination of B. necrophorus strains with 4 different antisera

STRAIN	DILUTIONS OF SERUM										0.1 PER CENT SALT SOLUTION + CULTURE CONTROL
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
						B. necrophorus A antiserum					
A	C	C	C	C	C	C	++++	+++	++	+	−
B	++++	++++	+++	++	+	+	+	+	+	+	+
C	C−	C	C	C	C	C	++++	+++	++	±	−
D	C	C	C	C	C	C	+++	++	+	±	−
E	C	C	C	C	C	C−	++++	++	+	±	−
F	C−	C−	C−	C−	C−	++++	+++	++	+	±	−
G	C	C	C	++++	++++	++++	+++	+++	++	+	±
H	++++	++++	++++	++++	++++	+++	+++	++	++	+	+
J	++++	++++	++++	++++	+++	++	+	+	±	−	−
						B. necrophorus B antiserum					
A	+++	++	+	+	+	+	+	+	+	+	+
B	C	C	++++	++++	++++	++++	++++	+++	++	+	+
C	−	−	−	−	−	−	−	−	−	−	−
D	++	+	−	−	−	−	−	−	−	−	−
E	−	−	−	−	−	−	−	−	−	−	−
F	+++	+	+	±	−	−	−	−	−	−	−
G	+++	++	++	++	+	+	+	+	+	+	+
H	+	+	+	+	+	+	+	±	±	±	±
J	±	−	−	−	−	−	−	−	−	−	−


B. necrophorus F antiserum
B. necrophorus J antiserum
A
B
C
D
E
F
G
H
J

agglutination affinities. With this in view a number of rabbits were immunized with various cultures. In most cases the treatment consisted of a series of injections given subcutaneously and intraperitoneally with a whole bouillon culture three days to a week old, which had been killed either by heating at 60°C. for one-half hour or by exposing to air for at least one day. The injections were made at intervals of three to five days and the doses increased gradually from 0.5 to 2 cc. Bleedings were made seven to eleven days after the last injection. Antiserum A used in the tests recorded in table 5 was obtained from a rabbit treated only with intravenous injections of culture filtrate from bouillon or of cooked meat media cultures nine to nineteen days old. Other lots of A serum from rabbits treated in the usual way with subcutaneous and intraperitoneal injections of killed whole bouillon cultures behaved in a similar manner. The other three sera (B, F, J) were also from rabbits injected subcutaneously and intraperitoneally with killed whole bouillon cultures. The sera prepared with the four different strains were tested for agglutinins against all the cultures. The results are given in table 5. Since some strains agglutinate spontaneously in normal (0.85 per cent) salt solution, the final dilutions were made in 0.1 per cent salt solution and a glucose digest bouillon growth was used as antigen.

The A antiserum agglutinates all strains at significant titers except Culture B. B antiserum, however, is relatively specific for B, while F antiserum agglutinates to a certain extent 7 of the strains and fails completely to agglutinate 2 cultures (C and E). The J serum agglutinates strongly only one culture (B) besides the homologous strain. It was noted that J and B, which agglutinate most poorly with A antiserum, agglutinate best with J antiserum, while others agglutinating well with A are not greatly affected by J.

According to the agglutination results, A seems to be an important strain since it is the only one among those used for producing antisera which stimulated the production of agglutinins for all strains. This result suggests that A antiserum possesses distinct agglutinins for all cultures. To test this, a complete series of agglutinin absorption tests were made between this antiserum and

each culture. Agglutinin absorption tests were also carried out with the other sera and a certain number of strains to see what other relationships could be established. The absorption was made in 1:10 dilutions of the serum in 0.2 per cent salt solution. Sediment obtained from centrifuging glucose digest bouillon cultures was added to the serum dilutions and the mixture incubated for 2 hours and then refrigerated. The next day, after centrifuging, agglutination tests were made with the absorbed serum. Some results of the absorption experiments with each serum are found in table 6.

The results of the complete absorption series with A antiserum indicate that A is a complex strain antigenically. It is apparently able to produce agglutinins for each strain, which in most cases can be removed only by absorption with A or with the particular strain under consideration. For instance, absorption by A takes out agglutinins for all strains. Strain G is apparently the next most complex culture antigenically, since absorption with this culture reduces to a considerable extent agglutinins for all strains except A and F and these are probably slightly reduced. Strains D, F, H, and J each remove from A antiserum chiefly agglutinins for themselves and leave agglutinins reacting with the other strains, although absorption by D causes a very slight reduction of agglutinins for a number of strains and the agglutinins for J are often decreased somewhat by absorption with other cultures. Strains C and E behave alike, in that each absorbs agglutinins for the other as well as for itself, and both leave agglutinins reacting with all other strains. This similar behavior of C and E agrees with their similarity in failing to agglutinate in the B and F antisera (table 5). Altogether these absorption results indicate that the power of the A antiserum to agglutinate all strains is apparently not due to some common group agglutinin, since, in general, the different strains fail to remove agglutinin for each other. On the other hand they suggest that the A antiserum possesses distinct agglutinins for each strain, since absorption by one heterologous strain removes chiefly agglutinins reacting with that particular strain and does not take out agglutinins for other heterologous strains except in special instances.

TABLE 6

Some results of absorption of sera by homologous and heterologous strains

ABSORBED BY	TESTED BY	DILUTIONS OF SERUM										0.1 PER CENT SALT SOLUTION CULTURE CONTROL
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
B. necrophorus A antiserum												
B	B	+	+	+	+	+	+	+	+	+	+	+
	A	C	C	C	C	C	C	+++	++	+	±	−
D	D	++++	++++	++	+	−	−	−	−	−	−	−
	A	++++	C	C	C	C	C	+++	++	+	±	−
F	F	++	+	±	±	±	±	±	±	±	±	−
	A	++++	C	C	C	C	++++	++	+	+	±	−
J	J	++	++	+	+	±	−	−	−	−	−	−
	A	++++	C	C	C	C	C	+++	++	+	+	−
A	A	++++	+++	++	++	+	−	−	−	−	−	−
B. necrophorus B antiserum												
A	A	−	−	−	−	−	−	−	−	−	−	−
	B	++++	++++	+++	+++	+++	+++	++	+	+	+	+
D	D	−	−	−	−	−	−	−	−	−	−	−
	B	++++	++++	++++	++++	++++	++++	++++	+++	++	+	+
F	F	−	−	−	−	−	−	−	−	−	−	−
	B	++++	++++	++++	++++	++++	++++	++++	+++	++	+	+
J	J	−	−	−	−	−	−	−	−	−	−	−
	B	++++	++++	++++	++++	++++	++++	++++	+++	++	+	−
B	B	+	+	+	+	+	+	+	±	±	±	±

B. necrophorus F antiserum

A	A	−	−	−	−	−	−	−	−	−	−	−
	F	C	C	C	++++	++	+	±	−	−	−	−
B	B	+	+	+	+	+	±	±	±	±	±	±
	F	++++	++++	++++	++++	++++	+++	++	+	+	±	−
D	D	±	±	−	−	−	−	−	−	−	−	−
	F	C	C	C	++++	++++	+++	++	+	+	±	−
J	J	+	−	−	−	−	−	−	−	−	−	−
	F	++++	++++	++++	++++	++++	+++	++	+	+	±	−
F	F	+	+	+	±	+	+	+	−	−	−	−

B. necrophorus J antiserum

A	A	−	−	−	−	−	−	−	−	−	−	−
	J	+++	+++	+++	+++	++	+	−	−	−	−	−
B	B	++++	+++	+	+	+	+	+	+	+	+	+
	J	C	C	++++	++++	+++	+	−	−	−	−	−
F	F	−	−	−	−	−	−	−	−	−	−	−
	J	C	C−	++++	++++	++	±					
J	J	−	−	−	−	−	−	−	−	−	−	−

In the case of the other sera the absorption tests, as far as carried out, show that absorption by the homologous culture removes specific agglutinins, while absorption by heterologous strains, although removing agglutinins for the absorbing strain, fails to absorb the specific agglutinins (table 6). Thus, at least in the case of the four strains used to produce these antisera, each one shows strong specific agglutinin production. Also, by the absorption experiments, A was found to possess complex antigenic properties.

PATHOGENICITY OF B. NECROPHORUS FOR LABORATORY ANIMALS

These bovine strains of *B. necrophorus* are pathogenic for rabbits. One cubic centimeter of a bouillon culture injected subcutaneously usually produces a spreading necrosis of the subcutaneous tissues. The area becomes edematous and necrotic and the rabbits die in four to ten days. Strain B differs from the others in not causing death but only producing an abscess at the point of inoculation which heals after a number of weeks. Strain A which has been kept in the laboratory for about three and one-half years is still as virulent as when first isolated. This strain has been tested on mice and guinea pigs. A subcutaneous inoculation of 0.1 or 0.2 cc. of a bouillon culture into mice caused a spreading necrosis and the mice died in eleven to twenty-five days. The culture had less effect on guinea pigs, at most producing an abscess which healed after two to three weeks.

In addition to living cultures, culture filtrates have been tested. In mice, 1 cc. of filtrate injected intraperitoneally produced illness and sometimes death. In rabbits, 4 cc. of filtrate injected intravenously produced illness and frequently death within a few hours (11 out of 17 cases). If the rabbits survived they showed considerable loss in weight within twenty-four hours, for instance, from 70 to as much as 235 grams or from 5 to 12 per cent of their body weight, and sometimes the loss in weight continued for two or three days following the administration. Smaller doses of filtrate (2 cc.), intravenously injected, regularly made the rabbits sick for a number of hours and caused a definite loss in weight. Since similar doses of bouillon had little or no effect on rabbits,

this action of culture filtrates is apparently due to some toxic substance from the culture.

DISCUSSION

This bovine group of necrophorus cultures show similar morphological appearances. The characteristic beaded forms occurring in all strains are due to unstained areas in the filaments which give the appearance of spores. However, these are not considered to be true spores since they are not affected by spore stains and the cultures containing them are readily killed by heat, drying and exposure to air. Furthermore, these beaded forms are more numerous in young cultures than in old ones and the clear areas vary in size and arrangement in the rods. Thus the unstained areas differ from spores in every respect except their failure to stain. There is no good reason why they may not be regarded as vacuoles occurring in the rapidly growing filaments.

These bovine strains in general represent a uniform group in regard to their behavior in various culture media and in fermentation reactions. As to pathogenicity, the strains exhibit similar properties with the exception of one which is much less virulent than the others.

When considered from an immunological standpoint, sharp differences appear. Although Culture A produced agglutinins reacting with all strains (table 5) yet absorption of the serum with heterologous strains failed to remove the agglutinins reacting with Strain A (table 6); and, moreover, each heterologous strain in general absorbed only agglutinins for itself and left agglutinins reacting with other cultures, indicating that A is a complex strain antigenically which apparently produces a number of distinct agglutinins. The three sera produced by immunization with other cultures showed further differences by agglutinating individual strains to varying degrees. All antisera behave alike, however, in retaining the homologous agglutinins after absorption with heterologous strains. Apparently each strain shows an homologous specificity in agglutinin production and may or may not develop agglutinins reacting with other strains.

SUMMARY

Ten strains of *B. necrophorus* isolated from cows have been described. They are similar in morphology, in most cultural reactions, and in pathogenicity for rabbits with the exception of one strain. When compared serologically by agglutination reactions certain differences were readily apparent, since immune sera prepared with four different strains allowed varying degrees of cross agglutination and apparently no cross absorption of specific agglutinins. Each immunizing strain apparently produces specific agglutinins since they are not absorbed by any other culture. Also, as found in the case of the A culture, a strain may stimulate the production of distinct heterologous agglutinins. The fluffy type of colonies usually found by other workers has not been observed in these cultures, which in all cases form smooth, even, compact colonies in serum agar shake cultures.

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ETIOLOGY OF SPONTANEOUS PULMONARY DISEASE IN THE ALBINO RAT

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The albino rat is susceptible to a chronic and frequently fatal pulmonary disease. It is important for investigators, particularly in tuberculosis, to recognize this condition because the lesions may be readily confused with those of experimental tuberculosis.

The early stage of spontaneous pulmonary disease produces no observable change in the appearance or activity of the animals. There are snuffles, dyspnea and torpor in the advanced stage, and the presence of the pulmonary lesions at this time can be demonstrated by the x-ray.

GROSS PATHOLOGY

Abscess formation is the characteristic feature of the disease. The lungs usually present single or multiple small abscesses, distributed throughout one or several lobes. If the disease is confined to one lobe, it is generally the lower one. The abscesses may coalesce and ultimately destroy the normal lung tissue of one or more lobes. They contain soft, partly inspissated, thick, gray or greenish-gray pus. In some instances, the arrangement of smaller abscesses about a terminal bronchus simulates bronchiectasis. Fibrous adhesions, parietal and interlobar, were common. Empyema was not observed. The tracheo-bronchial lymph nodes were enlarged but not caseous.

Microscopically, the central portion of the lesions was devoid of cellular structure. Surrounding this was a zone of varying width composed largely of polymorphonuclear leucocytes. Outside the polymorph zone the lymphocytes and monocytes faded off into the normal pulmonary tissue.

The older and larger lesions showed a large caseous central area surrounded by a dense fibrous capsule.

BACTERIOLOGY

We obtained on culture a Gram-negative, motile, aerobic bacillus, and a Gram-positive, anaerobic coccus from each of sixteen animals.[1] In most instances the cocci and bacilli were identified in smears made directly from the abscess. The bacillus grew well when smeared on blood agar plates and incubated aerobically, but the simplest and most effective procedure was to incubate a piece of the diseased lung tissue in meat infusion broth covered with an albolene seal. Both the cocci and the bacilli grow readily in this broth. After forty-eight hours' incubation, a number of blood agar plates were streaked from the broth, and plates incubated, aerobically for the bacillus and anerobically for the coccus. Pure cultures were easily obtained by proper selection of colonies and replating. In this series two types of Gram-negative, aerobic, motile bacilli were found. The first type produced on blood-agar a fine, opaque, raised, non hemolytic colony.

FIG. 1. ROENTGENOGRAM OF THE LUNGS OF AN OLD ALBINO RAT, SHOWING SPONTANEOUS DISEASE

FIG. 2. PHOTOMICROGRAPH OF A SMEAR MADE FROM THE PUS OF A LUNG ABSCESS SHOWING FUSIFORM BACILLI (FONTANA STAIN)

FIG. 3. PHOTOMICROGRAPH OF SMEAR MADE FROM A PURE CULTURE OF THE COCCUS (GRAM STAIN)

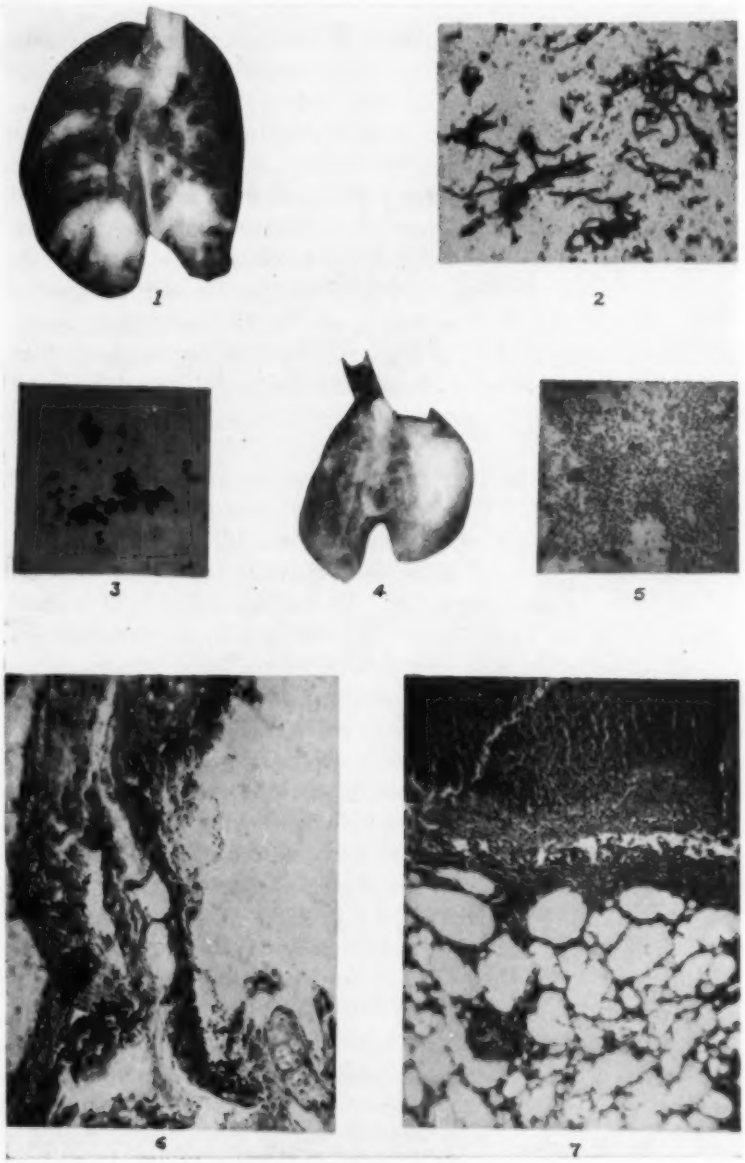
FIG. 4. ROENTGENOGRAM OF THE LUNGS OF AN ALBINO RAT, SHOWING THE DISEASE PRODUCED BY A MIXTURE OF PURE CULTURES OF THE COCCUS AND THE TYPE 2 BACILLUS

FIG. 5. PHOTOMICROGRAPH OF A SMEAR MADE FROM A PURE CULTURE OF THE TYPE 2 BACILLUS (GRAM STAIN)

FIG. 6. PHOTOMICROGRAPH OF A PATHOLOGICAL SECTION, SHOWING ULCERATION OF THE BRONCHIAL WALL SECONDARY TO AN EXPERIMENTAL SINUSITIS (HEMATOXYLIN AND EOSIN)

FIG. 7. PHOTOMICROGRAPH OF A PATHOLOGICAL SECTION, SHOWING THE WALL OF A PULMONARY ABSCESS PRODUCED BY INTRATRACHEAL INJECTION OF A MIXTURE OF THE COCCUS AND THE TYPE 2 BACILLUS (HEMATOXYLIN AND EOSIN)

[1] Sixteen albino rats with spontaneous pulmonary disease were obtained from the following sources: Four from Dr. H. C. Sherman's laboratory at Columbia University; two from a dealer in Philadelphia; two from Dr. E. V. McCollum's laboratory in Baltimore; and the remaining eight from our own stock.



FIGS. 1-7

Morphologically, this organism is a slender rod, 1.5 to 2μ in length containing polar granules. It is motile and Gram-negative. No liquefaction of gelatin, no acid formation or coagulation of litmus, milk, no formation of indol, and no reduction of nitrates was obtained. It does not produce acid or gas in carbohydrate media.

A moderate brownish growth is obtained on potato. Turbidity is readily produced in meat infusion broth. This bacillus conforms to the characteristics of *B. bronchisepticus*, which was first described by Ferry (Bergey, 1930, p. 365). Dr. Ruth Gilbert of the New York State Department of Health found that the organism was agglutinated in high dilution by a serum made from a strain of *B. bronchisepticus* isolated from a rabbit. This bacillus, associated with the anaerobic coccus, was obtained in 2 of the 16 rats in this series.

From the remaining fourteen rats we recovered a bacillus which produced on aerobic blood-agar plates a medium sized, opaque, raised, non-hemolytic colony, which grew more rapidly than the *B. bronchisepticus*, and at times became confluent and covered the entire surface of the plate. Morphologically it was a short, plump rod, 0.5 to 1μ in length, and contained polar granules (fig. 5). It was motile and Gram-negative. There was no liquefaction of gelatin, no acid or coagulation in litmus milk. Indol was formed and nitrates were reduced. Acid and gas were formed in the following carbohydrates: mannitol, lactose, galactose, inulin, dulcitol, xylose, raffinose, levulose, maltose, sucrose, glucose, and dextrin. It gave a fairly luxurious, brownish growth on potato. Turbidity was produced in meat infusion broth unaccompanied by an odor. *Aerobacter cloacae* (Bergey et al.) (*Bacillus cloacae*, Jordan) (Bergey, 1930, p. 333) corresponds to the characteristics of this bacillus except in the appearance of its colonies and in its fecal odor. Hereafter, this organism will be referred to as *Bacillus Type 2*.

From all sixteen of these animals we cultivated a coccus. The coccus when grown on an anaerobic blood agar plate produced a fine, discrete, pearly-white colony after forty-eight hours' incubation. There was no change in the blood agar be-

neath or around the colony. The organism was strongly Gram-positive and occurred singly or in pairs and rarely in short chains (fig. 3). No liquefaction of gelatin, no growth on potato, no acid formation or coagulation in litmus milk and no indol production was found. The organism reduced nitrate to nitrites, and gave a scant growth in meat infusion broth. It produced acid but no gas in mannitol, lactose, galactose, inulin, levulose, maltose, sucrose, glucose and dextrin.

It was observed that after several generations of anaerobic cultivation, the coccus would grow aerobically, and produce a rather luxurious growth similar to *Staphylococcus albus*. However, when re-inoculated into animals and cultivated again, it reverted to its original anaerobic characteristics.

REPRODUCTION OF THE DISEASE

Attempts were made to reproduce the disease by the following methods: (1) Intratracheal injection of pus taken directly from an abscess. (2) Intratracheal injection of pus from the nasal sinus of a rat suffering from "snuffles." (3) Intratracheal injection of a pure culture of the bacillus. (4) Intratracheal injection of a pure culture of the coccus. (5) Intratracheal injection of a mixture of the bacillus and coccus.

1. We made an emulsion in saline of some of the pus obtained from the first abscess studied, and then injected 0.2 to 0.4 cc. of this material into the trachea of each of five young rats. Smears and cultures from this material showed that it contained an anaerobic coccus and a Gram-negative aerobic bacillus (Type 2). One rat died after twenty-four hours and the necropsy revealed that the entire lung on one side was consolidated and red. The same coccus and bacillus were recovered from the lungs. After forty-eight hours another animal died of pneumonia, and the culture yielded the same combination of organisms. The other three were killed after two weeks and all had isolated abscesses, resembling the original. From all these abscesses, the same organisms were recovered. Three other animals were inoculated with pus from one of these experimental lesions. At necropsy ten days later, two of the three rats presented pulmonary abscesses, containing the same organisms.

2. We attempted to discover if possible, the source of the bacteria producing the pulmonary disease. Smears from the nasal sinuses of the rats in nearly every instance showed the same organisms found in the lung. Two rats were injected intratracheally with pus from the infected nasal sinuses. One of the animals developed a lung abscess. These observations lead to the hypothesis that the sinusitis was the primary infection and that the pulmonary disease was secondary by aspiration. We induced a purulent sinusitis in two normal rats with a mixed culture of the

TABLE 1

Intratracheal injection

MATERIAL USED	NUMBER OF RATS USED	ABSCESS	PNEUMONIA	BRONCHITIS	HEALED	NO RESULT
Pus direct from lung abscess containing bacilli and cocci	8	5	2	0	0	1
Broth culture of pus from lung abscess containing bacilli and cocci and fusiforms	3	1	0	0	0	2
Mixture of pure culture of bacilli and cocci	17	8	1	1	1	6
Pure culture of bacillus alone	6	0	0	0	0	6
Pure culture of coccus alone	6	0	0	0	0	6
Pus direct from nasal sinus	2	1	0	0	0	1
Total	42	15	3	1	1	22

anerobic coccus, the Type II bacillus and a fusiform bacillus which had been recovered from a previous spontaneous lung abscess in a rat. One animal died after two days and at necropsy the trachea and bronchi were filled with pus and there was extensive ulceration of the bronchi (fig. 6). All three organisms were identified in smears and sections of the lesion.

3, 4. In order to determine whether either one of the organisms isolated was alone capable of producing the disease, 0.5 cc. of a heavy saline emulsion of a pure culture of the Type 2 bacillus was injected intratracheally into each of 6 rats; and a similar amount of a pure culture of the coccus in the same manner was injected into another 6 rats. No disease was present at necropsy after

ten days in either series, with the single exception of an animal receiving a supposedly pure culture of the bacillus. In this instance smears and cultures revealed both a bacillus and a coccus.

5. Seventeen young rats, which showed no Roentgenographic evidence of lung disease, were inoculated intratracheally with a mixture of the anerobic coccus and Type 2 bacillus. Two animals were killed nine days later. Pneumonia was present in one and a purulent bronchitis in the other. The other fifteen rats were killed about three weeks later; in eight, acute pulmonary abscesses of varying sizes, which were observed in their gross and microscopical appearance were identical with those occuring spontaneously (fig. 7). One had a lesion at the base which had healed by fibrosis. Six showed no pulmonary disease (table 1).

Thirty mice were used to determine the relative pathogenicity of the coccus and the bacillus. We injected 0.25 cc. of a pure culture of the coccus into the groin of ten mice and 0.25 cc. of a pure culture of the bacillus into ten mice and a similar amount of a mixture of the coccus and bacillus into ten others. The animals receiving the pure culture of the coccus or the bacillus showed a temporary swelling in the groin which usually resolved within a week. The animals receiving the mixture showed a much larger lesion, from which the animal either died or presented at necropsy a chronic abscess. In only one case did the disease caused by the mixture, resolve itself.

RELATION OF SPONTANEOUS DISEASE TO EXPERIMENTAL TUBERCULOSIS

We inoculated ten rats in the groin and five rats intraperitoneally with bovine tubercle bacilli (B I culture Saranac Lake). Two of the first series and one of the second showed at necropsy thirteen months later a pulmonary disease in which tubercle bacilli were present. Pus from these lesions inoculated into a guinea pig produced tuberculosis. Sections made from the lungs of the rats showed giant cells containing large numbers of tubercle bacilli. However, cultures from the lesions also showed the presence of an anaerobic coccus and the Type 2 bacillus in addition to the tubercle bacilli. Five other animals, three of which re-

ceived injection of tubercle bacilli in the groin and two in the peritoneum, developed a pulmonary disease which contained only the spontaneous abscess organism. In no cases were the tubercle bacilli found alone.

Subsequent experiments on a strain of rats obtained from the original Wistar Institute stock have shown the presence of spontaneous lung disease containing the coccus and Type 2 bacillus. Fifteen of these rats were inoculated in the groin with bovine tubercle bacilli and given for three months the diet used by Grant (1926). At necropsy, five rats showed pulmonary lesions from which the coccus and Type 2 bacillus were recovered as well as the tubercle bacillus; four showed only the coccus and Type 2 bacillus; the remaining six were normal.

Lange (1925) in studying the effects of diets upon experimental tuberculosis, mentioned the prevalence of lung abscess and bronchiectasis in the albino rats used. Smith (1924 and 1926) and Grant (1926) have reported experiments on albino rats inoculated in the groin with tubercle bacilli and given a diet deficient in vitamins for a period of six months to one year. At necropsy, pulmonary disease in which the tubercle bacilli could be demonstrated was found to exist. They did not mention the presence of spontaneous, non-tuberculous lung abscess existing simultaneously with the tuberculous lesion.

Our results indicate that when albino rats are used for experiments in pulmonary tuberculosis spontaneous disease should always be considered.

DISCUSSION

The literature dealing with spontaneous lung abscess in the rat is rather meagre. Klein (1903) described a Gram-positive bacillus in two cases of rat pneumonia in 1903. This organism he named *Bacillus Muris*. Mitchell (1912) in 1912 found the same organisms in an outbreak of pneumonia in albino rats. Tunnicliff (1916) reported a Gram-negative streptothrix in 20 rats in 1916. In 1919 Hoskins and Stout (1919–20) obtained *B. bronchisepticus* from the upper respiratory tract and the lungs of rats suffering from respiratory disease. Jones (1922) described an

organism resembling *Bacillus actinoides* which he isolated from the lungs of 11 rats. He also found *B. bronchisepticus* in a few cases and in still others cocci were found.

McCordock and Congdon (1924–25) noted a small, Gram-negative motile bacillus in the pus found in otitis media in the albino rat, the descriptions, so far as given, corresponding to the Type 2 bacillus. Meyer and Batchelder (1926) studied a disease, pasteurellosis, in wild rats previously observed by Jonescu (1922) in three albino rats. A Gram-negative, bipolar, non-motile organism was described, which was virulent for rats only when injected in combination with streptococci.

In our animals (16) we found an anaerobic coccus in every case. In two instances it was associated with *B. bronchisepticus*; in 10 other animals, the coccus was associated with an unidentified bacillus called in this paper Type 2. In two animals, which had received thirteen months previously an injection of bovine tubercle bacilli in the groin, we found the coccus and the Type 2 bacillus as well as the tubercle bacillus. In one other case we found the coccus, the Type 2 bacillus and a fusiform bacillus present both in the nose and in pulmonary lesions and in still another rat, the coccus, the Type 2 bacillus, the tubercle bacillus and the fusiform were present.

It is obvious from our work and the review of the literature that there is no one common etiological agent in the lung disease of the albino rat. Apparently certain stocks of rats have one group of organisms and others have different types.

It is also apparent that the presence of tubercle bacilli in the lungs after a previous inoculation in the groin or the peritoneum is not proof that the pulmonary disease is primarily tuberculosis, unless cultures are made to eliminate the possibility of the presence of other organisms commonly found in pulmonary disease in albino rats.

CONCLUSIONS

1. A number of different types of organisms are capable of producing pulmonary infections in the albino rat, such as *Bacillus actinoides, Bacillus bronchisepticus, Bacillus muris*, and a streptothrix.

2. In a group of sixteen rats we studied there were present (1) an unidentified anaerobic coccus, in all cases; (2) an unidentified aerobic Gram-negative bacillus in fourteen cases associated with the coccus; (3) in two cases *Bacillus bronchisepticus* was associated with the coccus; and in two others (4) a large fusiform bacillus was also present, which morphologically was identical with the Vincent's fusiform found frequently in the lung abscesses in man.

3. A symbiosis of the unidentified coccus and the unidentified bacillus was demonstrated by our failure to reproduce the disease by inoculation of pure cultures of either, while the combined pure cultures produced a lesion identical with the spontaneous disease.

4. In experiments with tubercle bacilli in albino rats, care should be taken to exclude the possibility of the presence of a co- or pre-existing spontaneous lung disease in which the tubercle bacillus may become implanted.

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PEROXIDASE IN RELATION TO BACTERIAL GROWTH WITH SPECIAL REFERENCE TO THE INFLUENZA BACILLUS

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The most interesting work of recent years on *Bacillus influenzae* has dealt with its growth requirements. A number of investigators (Davis, 1921 a and b; Fildes, 1921; Rivers and Poole, 1921; Thjötta and Avery, 1920–1921) have definitely shown that two distinct accessory food substances or factors are essential to the growth of true influenza bacilli in aerobic culture. Thjötta and Avery (1920–1921) designated the two separable factors as X and V respectively. Since the recognition and separation of the two factors, organisms have been described which require only one of the factors for their growth in aerobic culture. Rivers (1922, a, b, and c) reported that Friedberger's *Bacillus hemoglobinophilus-canis* requires the X-factor only, and that bacilli have been obtained from human cases which require the V-factor only. The latter were called *Bacillus parainfluenzae*.

The nature of the X and V factors and their rôle in the growth of the influenza bacillus has not been made clear. For the most part the cultivation of the influenza bacillus has been associated with the presence of peroxidase in the medium. Olsen (1921) states that media which supported its growth gave a positive guaiac or benzidine test for peroxidase. Fildes (1921) believes that peroxidase is a dominant factor in the growth of the influenza bacillus and appears to be identical with the X-factor. The work of Fildes (1924) in which the V-factor organism grew in symbiosis with the X-factor organism on ordinary media, indi-

cates that those organisms which require for growth one factor only in the culture medium, either X or V, synthesize the other factor. On this basis it was assumed that, if the X-factor were peroxidase, then the V-factor organism which does not require for growth the addition of X-factor to the medium would give a positive peroxidase test, whereas, the X-factor organism which requires the addition of X-factor and the true influenza bacillus which requires the addition of both factors to the medium would give a negative peroxidase test. After undertaking to establish the truth of this hypothesis and obtaining the results of this investigation, search of the literature revealed that Valentine and Rivers (1927) had found previously that the V-factor organism in mass gave a positive peroxidase test. Therefore, this investigation is merely an elaboration of the work begun by Rivers.

Kopp (1927–1928) published a paper on the growth requirements of the influenza bacillus in regard to the X and V factors in anaerobic culture. Under anaerobic conditions he found that the true influenza bacillus does not require the X-factor for growth, only the V-factor being essential. His results have been corroborated recently by the writer.[1] The growth of the influenza bacillus, anaerobically, on peroxidase-free media in the form of V-factor agar or broth, afforded a means of determining whether the organism produced peroxidase. Previous to Kopp's discovery there was no satisfactory way of testing the bacillus for peroxidase production since it was grown on media which themselves gave a positive peroxidase reaction.

In this study eight strains were employed. They included 6 strains (one of which was hemolytic) of true influenza bacilli, 1 strain (No. 2) of *Bacillus parainfluenzae* (V-factor organism) and 1 strain (No. 8) of *Bacillus hemoglobinophilus-canis* (X-factor organism). None of the strains grew on plain agar aerobically. All of the strains grew anaerobically on agar containing V-factor alone. The eight strains were grown anaerobically on V-factor agar for forty-eight hours at 37°C. and tested for the production of peroxidase by the benzidine method.

[1] Journal of Hygiene, in press.

Reagent. A large pinch of crystalline benzidine-hydrochloride was placed in a test tube with 3 cc. of glacial acetic acid. After most of the crystals had dissolved, an equal volume of hydrogen peroxide was added. A peroxidase-containing substance in contact with this milky white reagent turns blue.

TABLE 1

The production of peroxidase by true influenza and influenza-like organisms

STRAINS	HEMOLYSIS	FACTORS REQUIRED AEROBIC CONDITION	PEROXIDASE
1	+	X and V	−
2	−	V	+
8	−	X	−
27S	−	X and V	−
44	−	X and V	−
121	−	X and V	−
126	−	X and V	−
499	−	X and V	−

TABLE 2

Production of peroxidase by anaerobes as shown by the benzidine reaction

ANAEROBES	PEROXIDASE PRODUCTION
1. *B. aerofoetidis*	−
2. *B. bellonensis*	−
3. *B. bifermentans*	−
4. *B. botulinus* A	−
5. *B. botulinis* (European)	−
6. *B. fallax*	−
7. *B. oedematis*	−
8. *B. putrificus*	−
9. *B. sporogenes*	−
10. *B. tertius*	−
11. *B. tetanus*	−
12. *B. vibrion septique*	−
13. *B. Welchii*	−

A drop of the reagent was placed on the colonies produced by each strain. The true influenza bacilli and *Bacillus hemoglobinophilus-canis* gave negative reactions. *Bacillus parainfluenzae* gave a positive reaction, in which the individual colonies turned a

solid blue color. The parainfluenza organism exhibited the positive reaction for peroxidase on V-factor media both aerobically and anaerobically in successive transplants. Table 1 indicates the results obtained with each strain.

Avery and Morgan (1923–1924) found that an iron compound which gave the benzidine reaction for peroxidase when added to

TABLE 3

The production of peroxidase by aerobes

AEROBES	PEROXIDASE REACTION	AEROBES	PEROXIDASE REACTION
1. *Act. asteroides*	+	23. *B. subtilus*	+
2. *B. acidi-lactici*	+	24. *B. suipestifer*	+
3. *B. alkaligenes*	+	25. *B. typhosus*	+
4. *B. anthracis*	+	26. *B. xerosis*	+
5. *B. anthracoides*	+	27. *Br. melitensis*	+
6. *B. cereus*	+	28. *M. tetragenus*	+
7. *B. coli-communior*	+	29. *Myc. stercusis*	+
8. *B. coli-communis*	+	30. *Ps. fluorescens*	+
9. *B. diphtheriae*	+	31. *Sarc. aurantiaca*	+
10. *B. fuchsina*	+	32. *Sarc. lutea*	+
11. *B. Hofmanni*	+	33. *Sarc. ventriculi*	+
12. *B. lactis aerogenes*	+	34. *Sp. cholerae*	+
13. *B. mallei*	+	35. *Sp. Finkler-Prior*	+
14. *B. Morgani*	+	36. *Sp. Metchnikovi*	+
15. *B. mycoides-roseus*	+	37. *Sp. tyrogenum*	+
16. *B. paratyphosus* A	+	38. *Staph. albus*	+
17. *B. pestis-caviae*	+	39. *Staph. aureus*	+
18. *B. prodigiosus*	+	40. *Staph. citreus*	+
19. *B. proteus-vulgaris*	+	41. *Staph. zymogenes*	−
20. *B. pseudotetanicus*	+	42. *Str. bovis*	−
21. *B. pyocyaneus*	+	43. *Str. liquifaciens*	−
22. *B. rouge de Kiel*	+	44. *B. suisepticus*	−

bouillon supported the growth of obligate anaerobes in the presence of air through repeated transfers. In view of this fact and the results with the influenza bacillus and related organisms which suggest that the function of peroxidase is intimately connected with the ability of these strains to grow in the presence of air, several anaerobes were tested for peroxidase. They were transferred to fresh meat tubes and incubated for forty-eight hours at

37°C.; a drop of the meat culture was transferred by a capillary pipette to an infusion agar plate and streaked with a platinum loop. After seventy-two hours incubation in the anaerobic jar, the strains were tested for peroxidase by transferring a loop of the plate culture to a drop of the benzidine reagent. Of thirteen anaerobes tested, not one gave a positive reaction for peroxidase. The results are shown in table 2.

Since the influenza bacillus has been grown on agar medium in symbiosis with certain aerobic organisms it was decided to test a representative number of aerobes for the production of peroxidase. The tests were carried out in the manner described for anaerobes, i.e., a loop of culture, from plain or infusion agar medium, of each strain was transferred to a drop of the benzidine reagent. Of 44 aerobes tested only 4 were negative for peroxidase. Table 3 summarizes the results. Organisms such as the diphtheria bacillus and xerosis bacillus which, from reports of various investigators, enable the influenza bacillus to grow well in symbiosis on blood-free media were especially rich in peroxidase as revealed by the intensity of the color reaction with benzidine. It is of interest to note that Williams and others (1914) were unable to obtain the growth of influenza strains in the presence of streptococci on blood-free media. Table 3 shows the streptococci which were examined, negative in regard to peroxidase. The same may have been true for Williams' streptococci which failed to induce growth. Thus, the growth of influenza bacilli in association with aerobes does not eliminate the need for the presence of peroxidase.

Thjötta and Avery (1921) stated that banana contains the essential growth factors but fails to react positively to benzidine. If this is so, then it would be an exception to the rule that peroxidase is essential to the growth of the influenza bacillus in aerobic culture. Banana was tested in this study for peroxidase and surprisingly gave a positive reaction to benzidine. It appears then that the influenza bacillus has never been grown aerobically without the presence of peroxidase in the medium.

The question arises whether there is a functional difference between the action of the very stable peroxidase-like substance associated with an iron compound in hemoglobin, and the com-

paratively heat labile peroxidase found in vegetable tissues. Opinions differ in this regard.

Madelung is of the opinion that the activation of peroxides by hemoglobin is functionally not different from their activation by tissue peroxidases as there apparently exist in the tissues complex iron compounds which may be capable of conveying oxygen. On the other hand, Oppenheimer states, that for the present at least we must accept the active, thermo-labile peroxidases as ferments. The fact should be recognized that we can demonstrate very active peroxidases not containing manganese or iron. In the living tissues provisions would seem to be made that biological catalyzing agents, ferments, should do what under other conditions inorganic catalyzers are able to do.[2]

In view of the latter fact it would seem that the peroxidase-like activity of blood and of known chemical compounds which function as the X-principle in relation to the influenza bacillus is that of an inorganic catalyst, while the tissue peroxidases which function as the X-factor are analogous to organic catalysts, with both types of catalysts performing the same function.

DISCUSSION

Although it is definitely known that the influenza bacillus will grow anaerobically without the X-factor, which all evidence indicates to be peroxidase, the nature of its action in aerobic cultures still remains to be explained.

In Callow's work (1923) on catalase in bacteria, none of nine anaerobes tested gave off gas when treated with hydrogen peroxide, whereas, all of the aerobes investigated were shown to contain catalase under both aerobic and anaerobic conditions with the exception of the streptococci.

These results, as far as they go, show a striking parallelism to the peroxidase test carried out in the present study. Accordingly, the non-peroxidase-producing organisms fall into three main groups, (1) that represented by the anaerobes which fail to grow aerobically on solid media even though peroxidase has been added, (2) that represented by the influenza bacilli which grow aerobi-

[2] Quoted from von Fürth, Chemistry of Metabolism, Smith.

cally in liquid or on solid media upon the addition of peroxidase when the second factor is also present and, (3) that represented by certain streptococci which neither produce peroxidase nor require it for growth.

Callow states,

> According to Wieland's theory of respiration, water is decomposed by an oxido-reductase into (OH) and (H), the latter combining with the oxygen of the air to form hydrogen peroxide which is at once decomposed by the enzyme catalase into water and molecular oxygen. In the absence of catalase the hydrogen peroxide would accumulate and kill the cell. . . . . In the absence of air no hydrogen peroxide would be formed and anaerobes would be able to develop.

While growth of anaerobes aerobically on solid media in the presence of peroxidase does not occur, Avery and Morgan (1923–1924) have reported their growth through repeated transfers in broth containing an iron compound with the usual reactions of peroxidase. Callow (1923) on the other hand, obtained no conclusive evidence that anaerobes were able to grow in broth containing catalase prepared from bacteria, fat and yeast. The only difference recognized between the terms catalase and peroxidase seems to be that the former is thought to liberate molecular oxygen and the latter atomic oxygen from hydrogen peroxide.

Applying McLeod's and Gordon's theory of hydrogen peroxide production by bacteria, the difference in the growth requirements of the three groups mentioned would appear to involve its production and their sensitiveness to it. According to these authors, it may be supposed that all bacteria tend to produce hydrogen peroxide and that the production of catalase by the majority of bacteria, prevents the accumulation of hydrogen peroxide to which they are only slightly or moderately sensitive. On the other hand they state that the sensitiveness of anaerobes to hydrogen peroxide, which they produce as soon as oxygen is available, is so very great that they cannot tolerate minute traces even in the presence of added catalase which they lack, therefore they cannot grow or survive in the presence of oxygen. The influenza bacilli would apparently belong to a group whose sensitiveness

to hydrogen peroxide is not great enough to prevent growth under aerobic conditions when the missing peroxidase is artificially supplied in the medium. To quote McLeod and Gordon,

> Against the general application of such a theory is the group (three) which is apparently devoid of catalase [and peroxidase], "not particularly sensitive to hydrogen peroxide and have not been shown to produce hydrogen peroxide. An alternative supposition would be that the production of hydrogen peroxide is associated with a certain type or types of bacterial metabolism.

It could still be assumed that the third group produces hydrogen peroxide but in such minute amounts as not to be detected and to which its sensitiveness is negligible; in such a case catalase or peroxidase would not be required. However, we are still left in the dark as to the actual conditions.

Whatever definite rôle the X-principle of peroxidase plays in the growth of the influenza bacillus, it must be concerned in rendering harmless an otherwise harmful substance formed in the presence of atmospheric oxygen. It seems unlikely that molecular oxygen as such is directly harmful,—else how could peroxidase act to make it harmless?

CONCLUSIONS

1. *Bacillus influenzae*, hemolytic and non-hemolytic and *Bacillus hemoglobinophilus-canis* do not produce peroxidase. *Bacillus para-influenzae* produces peroxidase under both aerobic and anaerobic conditions.

2. Not one of 13 anaerobes tested, reacted positively to the benzidine test for peroxidase.

3. Of 44 aerobes tested, all but four strains, two of which were streptococci, gave a positive reaction for peroxidase.

4. Banana contains peroxidase as tested by benzidine.

5. All evidence appears to substantiate Fildes' view (1922) that the X-factor is associated and identical with peroxidase.

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A SYSTEM FOR THE BACTERIOLOGICAL EXAMINATION OF WATER

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INTRODUCTION

A tremendous literature has accumulated during the last several years on methods for the bacteriological examination of water. The subject has attracted considerable interest chiefly from the standpoint of the interpretation of results and, also, with a view to the elimination of false or spurious positive presumptive tests, with the aid of various dyes incorporated into the media.

Organisms which were at one time classed as unsafe are now no longer held, by the majority of workers, to have the same sanitary significance. In other words, the colon group is now divided into the coli and aerogenes sections of the colon group.

The elimination of false or spurious presumptive tests with the aid of various dyes incorporated into the media has occupied the attention of many investigators. This is a very important forward step as it not only makes the results more clear cut, but effects a great saving in time and materials. The need at the present time is to obtain results as quickly as possible and the addition of dyes to the media is a step in this direction.

Considering all of the newer methods being advocated, the majority of workers have failed to appreciate the fact that the borderline or atypical strains are not eliminated. We are no nearer to a solution of these atypical strains now than we have ever been. It is relatively easy to identify a typical *B. coli* but a very difficult matter to classify an atypical strain. It is not

believed that a medium will ever be devised to entirely eliminate these borderline organisms but if many or most of them are classed as colon or not colon a great step forward will have been made.

Unfortunately many of the procedures have tended to complicate rather than simplify the subject. The need at present is to simplify the methods as much as possible and at the same time produce the desired results.

A complete system for the bacteriological examination of water is here presented. It is believed that the method will not only prove simple and effect a great saving in time and materials, but that the atypical or borderline strains will be reduced to a minimum. The method has already been shown to possess great possibilities, proving far more satisfactory to the writer than any now in use.

A NEW MEDIUM FOR THE PRESUMPTIVE TEST

Churchman (1912) as early as 1912 showed that gentian violet in a concentration of 1:100,000 exhibited a marked inhibitory action on the Gram positive organisms but that it had very little, if any, effect on those organisms which stained Gram negatively. This applies not only to vegetative cells but also to bacterial spores, although Churchman (1912), Burk and Skinner (1925), Wiesner (1926), and others have shown that a higher concentration of dye is necessary. Churchman stated that the above effect is better described as bacteriostatic rather than bactericidal because all organisms are not necessarily killed but merely prevented from multiplying. These results have been further verified in subsequent papers by the same author (Churchman, 1921, 1922 and 1923) by Gay and Beckwith, (1922), and others. This same principle has been made use of by Petroff (1915) in the isolation of the tubercle bacillus from sputum and feces; by Krumwiede, Fielder and Watson (1918) for the purification of vaccine virus; by Bernstein and Loewe (1919) for the isolation of the influenza bacillus, and by many others for various special investigations.

Various dyes are being used more and more for the elimination

of positive presumptive tests that do not confirm for the members of the colon group. These false tests are attributed to three causes: (1) bacterial symbiosis, (2) aerobic spore formers, and (3) anaerobic spore producers. Elimination of the above three causes means a saving of a considerable amount of time in obtaining results.

Bacterial symbiosis

By bacterial symbiosis is meant the production of acid and gas from carbohydrate media by two organisms living together, neither of which acting alone is capable of producing gas. This phenomenon which was first investigated by Sears and Putnam (1923) has actually been found to be the cause of many false presumptive tests. Others who have brought forth convincing evidence as to the existence of bacterial synergism are Leitch (1925), Castellani (1926), and Holman and Meekison (1926). Dyes have been very useful in the elimination of the majority of spurious tests due to bacterial symbiosis.

Spore formers

Aerobic spore bearing bacilli have been responsible for many positive presumptive tests which have failed to confirm for the presence of *B. coli*. Ewing (1919) isolated a spore bearing organism which was able to produce acid and gas in lactose. Similar organisms have been isolated by Norton and Weight (1924), Sohn (1925), Hostettler and Gettrust (1926), and Koser and Shinn (1927).

Meyer (1918) repeatedly recovered an anaerobic spore forming organism giving gas from lactose broth. Levine (1918), Frost (1916), Kahn (1918), Muer and Harris (1924), Raab (1923), Meader and Bliss (1923), Norton and Barnes (1928), Spray and Laux (1930) and others also reported on the presence of anaerobic organisms interfering with the presumptive test.

The use of dyes

Brilliant green, gentian violet and crystal violet have been used more than any other dyes for the elimination of Gram

positive organisms. These dyes in high dilution are particularly effective against those organisms which are not decolorized by alcohol, but, on the other hand, show a minimum of effect on the Gram negative bacteria. Of the three dyes, brilliant green has probably been used more in water works practice.

Brilliant green was originally introduced into bacteriological technique for the isolation of the typhoid bacillus from feces and urine. Browning, Gilmour and Mackie (1913) found that this dye exerts a much more marked inhibitory effect on *B. coli* than on *B. typhosus* or the paratyphoid organisms. In the presence of brilliant green the typhoid bacillus is not outgrown by the colon organisms. This was further shown by Browning and Gilmour (1913), Krumwiede and Pratt (1914), Browning, Mackie and Smith (1914), Krumwiede, Pratt and McWilliams (1916), and Teague and Clurman (1916a). Teague and Clurman (1916b) used brilliant green for preserving typhoid stools for delayed examinations.

Bile-dye media

Bile or its salts, either with or without dyes, are extensively used in sugar broth media for their stimulating effect on the growth of the colon organisms. Jackson (1906) employed lactose bile for the presumptive test. He concluded that the bile broth prevented negative tests from the overgrowth of other bacteria as well as positive tests from mixtures of other gas formers. Levine (1921) found that in a medium containing 0.5 per cent peptone with crystal violet, a bile concentration less than 0.5 per cent stimulated growth of *B. coli* while more than that amount exhibited a marked inhibitory action. Similar results were obtained when bile salts were used (Levine, 1922). The amount of bile to be added varied with the concentration of peptone. In the presence of bile or its salts a higher concentration of dye is necessary, due to absorption and inactivation. According to Dunham, McCrady and Jordan (1925) the following deductions can be made as to the use of brilliant green bile broth: (1) Its use eliminates the large number of confirmatory plates on which

no growth occurs; (2) spore forming organisms are eliminated; (3) brilliant green bile appears to extend its selective inhibitions to attenuated forms of the colon group. Other workers who have reported favorably on the use of brilliant green bile broth are Ruchhoft (1926), Dunham and Schoenlein (1926), Hale (1926), Hinman (1927), and Jordan (1927). Hall and Ellefson (1918 and 1919) used gentian violet without bile for the elimination of spurious presumptive tests. The use of gentian violet has also been advocated by Wagner and Monfort (1921) and Stearn (1923). Dominick and Lauter (1929) used methylene blue without bile. On the other hand Howard and Thompson (1925) found that brilliant green bile broth is highly inhibitory and useless except to restrict the growth of the colon group. This applied not only to the use of whole bile but also to bile salts. Poe (1930) reported that crystal violet alone, would eliminate most anaerobes. With most strains of *B. welchii* the addition of bile actually stimulated their growth. The inhibitive power of the crystal violet lactose broth was destroyed by the addition of bile, as far as the growth of anaerobes was concerned.

Choice of dyes

It is rather unfortunate that gentian violet was ever used in bacteriological media to restrict the growth of certain organisms. Gentian violet is a term which does not refer to any definite chemical compound but rather to a mixture of dyes of a certain group (Committee, 1922). Grübler apparently originated the term. It is not recognized in the dye or textile industry. Such dyes in the mixtures sold as gentian violet all belong to the pararosaniline series but at the present time the different manufacturers and dealers nearly all sell different mixtures of these dyes under the name of gentian violet. On the other hand, crystal violet, which is one of the components of gentian violet is a definite chemical compound. A definite chemical formula can be assigned to this dye and, although commercial samples of it are not necessarily pure and may vary in their composition, it should be used in preference to gentian violet.

Experimental

Brilliant green is used to a far greater extent for its inhibitory action on the growth of Gram-positive organisms than crystal violet. It is not clear just why this dye has been selected for this purpose in preference to the latter. Browning, Gilmour and Mackie (1913) in the course of systematic investigations on the antiseptic action of benzol derivatives found that a marked difference existed between the action of dyes which are members of the diamidotriphenyl-methane group (brilliant green) and those of the triamidotriphenyl methane series (crystal violet). Both groups exert a powerful action on Gram-positive organisms. In addition, the diamidotriphenyl methane dyes, of which brilliant green is a member, are also fairly actively bactericidal toward the coli-typhoid group, whereas these organisms are comparatively insusceptible to the triamidotriphenyl methane group (crystal violet).

Comparative studies on dyes incorporated in media and inoculated with several organisms strengthen the findings of Browning, Gilmour and Mackie, that brilliant green is more bactericidal than crystal violet towards the colon organisms. In table 1 are results obtained when various dilutions of crystal violet and brilliant green are inoculated with *B. coli*, *B. aerogenes*, *Staphylococcus albus* and *Staphylococcus aureus*, and *B. subtilis*. All tubes were examined at the end of forty-eight hours for turbidity. With crystal violet it can be seen that *B. coli* is relatively insusceptible to its action as compared with brilliant green. On the other hand crystal violet exerts a more toxic effect against the Gram-positive organisms than brilliant green. In other words, a greater range of safety exists between Gram-positive and Gram-negative organisms when crystal violet is used. More crystal violet can be used than brilliant green without exerting any more toxic action on *B. coli*. This means that with crystal violet a better chance exists for the elimination of those Gram-positive organisms, responsible for false tests, than when brilliant green is used.

The amount of dye to be incorporated varies with the amount

TABLE 1

Comparison of bactericidal action of crystal violet and brilliant green

Medium employed:

Peptone, Difco	5 grams
K_2HPO_4	5 grams
KH_2PO_4	1 gram
Lactose	5 grams
Dye, sufficient quantity	
Distilled water, to make 1000 cc.	

DYE DILUTION	CRYSTAL VIOLET					BRILLIANT GREEN				
	B. coli	*B. aerogenes*	*Staph. albus*	*Staph. aureus*	*B. subtilis*	*B. coli*	*B. aerogenes*	*Staph. albus*	*Staph. aureus*	*B. subtilis*
50,000	−	−	−	−	−	−	−	−	−	−
100,000	−	+	−	−	−	−	−	−	−	−
150,000	+	+	−	−	−	−	+	−	−	−
200,000	+	+	−	−	−	−	+	−	−	−
250,000	+	+	−	−	−	−	+	−	−	−
300,000	+	+	−	−	−	−	+	−	−	−
350,000	+	+	−	−	−	−	+	−	−	−
400,000	+	+	−	−	−	−	+	−	−	−
450,000	+	+	−	−	−	−	+	−	−	−
500,000	+	+	−	−	−	−	+	−	−	−
1,000,000	+	+	−	−	−	−	+	−	−	−
1,500,000	+	+	−	−	−	−	+	−	−	−
2,000,000	+	+	−	−	−	−	+	−	−	−
2,500,000	+	+	−	−	−	+	+	−	−	−
3,000,000	+	+	−	−	−	+	+	−	−	−
3,500,000	+	+	−	−	−	+	+	−	−	−
4,000,000	+	+	−	−	−	+	+	−	−	−
4,500,000	+	+	−	−	−	+	+	−	−	−
5,000,000	+	+	−	−	−	+	+	−	−	−
6,000,000	+	+	−	−	−	+	+	−	−	−
7,000,000	+	+	−	−	−	+	+	−	−	−
8,000,000	+	+	−	−	−	+	+	−	−	+
9,000,000	+	+	−	−	−	+	+	−	−	+
10,000,000	+	+	−	−	−	+	+	−	−	+
11,000,000	+	+	−	−	−	+	+	−	−	+
12,000,000	+	+	−	−	+	+	+	+	−	+
14,000,000	+	+	−	−	+	+	+	+	−	+
16,000,000	+	+	−	−	+	+	+	+	−	+
18,000,000	+	+	+	−	+	+	+	+	−	+
20,000,000	+	+	+	−	+	+	+	+	−	+
22,000,000	+	+	+	−	+	+	+	+	−	+
24,000,000	+	+	+	−	+	+	+	+	−	+

All readings were made at the end of forty-eight hours.

of peptone and other organic matter added to the media. As the amount of peptone is increased the amount of dye also is increased. In other words, the concentration of dye necessary to produce the desired results varies directly with the amount of peptone.

Using the medium as given in table 1, it was found that a dye concentration of 1:700,000 did not affect the growth of the colon organisms, and, at the same time was sufficient to eliminate the growth of organisms giving false presumptive tests.

Crystal violet broth

Preparation of crystal violet solution. Place 1 gram of crystal violet in a glass stoppered bottle of 150 cc. capacity and add 50 cc. of 95 per cent alcohol. Shake thoroughly and then add 50 cc. of distilled water. Shake well again until all of the dye is dissolved. Pipette 20 cc. of this 1 per cent solution into a liter volumetric flask. Add sufficient water to make 1000 cc. This gives a 1:5000 solution of crystal violet.

The medium for the presumptive test is now prepared as follows:

Peptone, Difco	15	grams
K_2HPO_4	15	grams
KH_2PO_4	3	grams
Lactose	15	grams
Crystal violet (1:5000)	21.5	cc.

Distilled water to make the quantity = 2000 cc.

Mix ingredients and stir until solution is effected. Distribute the medium into test tubes, 20 cc. to each tube. Sterilize in an Arnold sterilizer or in the streaming steam of an autoclave for ten minutes. Only one heating is sufficient to sterilize. Autoclaving under pressure will decompose some of the dye making the medium unfit for use. It is not necessary to adjust the reaction of the medium as this is controlled by the buffer salts.

The tubes are inoculated with a 10 cc. portion of water sample, giving a final volume of 30 cc. This brings the final volume of the two liters of medium to three liters. The formula after the addition of the water sample becomes:

Peptone, Difco	15	grams
K_2HPO_4	15	grams
KH_2PO_4	3	grams
Lactose	15	grams
Crystal violet (1:5000)	21.5	cc.
Distilled water to make	2000	cc.
Water sample	1000	cc.
	3000	cc.

It makes no difference which dilutions are run as long as the above proportions are not disturbed. All that is necessary is to increase or decrease the amount of distilled water used in preparing the medium.

As an example, if 1 cc. amounts are to be inoculated into tubes, this may be calculated as follows:

	cc.
Amount of medium per tube	4
Amount of inoculum per tube	1
Total	5

$$\frac{1}{5} : : \frac{x}{3000} \qquad 5x = 3000$$

$x = 600$ cc. = the inoculum

$3000 - 600 = 2400$ cc. = the medium

Prepare the above medium using sufficient distilled water to make 2400 cc. Distribute 4 cc. amounts in test tubes. Then, on inoculation with 1 cc. quantities of water sample, the above proportions of the various ingredients will be the same as in the tubes holding 10 cc. amounts of water sample.

Inoculating tubes of crystal violet broth with 50 strains of *B. aerogenes* and 180 strains of *B. coli* yielded the results shown in table 2.

TABLE 2

Results obtained by inoculating crystal violet broth medium with pure cultures of B. coli and B. aerogenes

ORGANISM	NUMBER OF STRAINS	10 PER CENT OR MORE GAS	CORRELATION
			per cent
B. aerogenes	50	50 positive	100
B. coli	180	180 positive	100

It is seen that perfect correlation is obtained by inoculating tubes of the medium with pure cultures of *B. coli* and *B. aerogenes*. A dye concentration of 1:700,000 is not inhibitory to the growth of these two organisms.

A NEW MEDIUM FOR THE CONFIRMATORY TEST

The majority of the solid media now in use for the detection of *B. coli* in water were originally used for the isolation of *Bacillus typhosus* and *Bacillus dysenteriae* from infected feces and urine. Changes have been made in their composition and mode of preparation adapting their uses to water works practice. Very few of the media now extensively in use were compounded solely for water examinations. This accounts for the large number of modifications of Endo and other differential media. This is a point that should be kept in mind.

Drigalski and Conradi (1902) used crystal violet to eliminate other organisms interfering with the isolation of *B. typhosus* from stools. Litmus was added to the medium to identify the presence of the acid producing colon organisms. The well known medium of Endo (1903) is composed of basic fuchsin decolorized by the use of sodium sulfite. Modifications of the original formula have been reported by Kendall and Walker (1910), Kendall and Day (1911), Kinyoun and Deiter (1912), Harding and Ostenberg (1912), Robinson and Rettger (1916), Levine (1918), and others adapting the medium to special investigations. Holt-Harris and Teague (1916), incorporated the dyes methylene blue and eosin in an agar base containing lactose and sucrose and were able to isolate the typhoid and dysentery organisms on this preparation. This medium has been modified and simplified by Levine (1918 and 1921), adapting its use to water works laboratories.

Differentiation of the coli and aerogenes groups

The consensus of opinion among bacteriologists at the present time is that the coli and aerogenes divisions of the colon group should be differentiated. This was advocated, as early as 1918, by Rogers (1918) and Levine (1918). These organisms are no

longer held to have the same sanitary significance in reporting on the potability of a water supply. It has been shown by Rogers and his associates (Rogers, Clark and Davis, 1914; Rogers, Clark and Evans, 1914 and 1915; Rogers, Clark and Lubs, 1918), that *B. coli* is characteristic of the intestinal tract of man and animals while *B. aerogenes* is very rarely isolated from human or animal excreta, but is found chiefly in soil and on grains. Therefore these two organisms should not be considered the same from a sanitary standpoint. Among others who believe the two organisms should be separated may be mentioned Wood (1919), Levine (1921), Wilson (1922), Hinman (1925), Clark (1925), Spray (1927), and Chen and Rettger (1920).

Rogers, Clark and Davis (1914), Rogers, Clark and Evans (1914 and 1915), and Rogers, Clark and Lubs (1918) in the course of extensive and systematic investigations on the coli and aerogenes organisms were able to separate the members into two main groups by means of their gas ratios. The members of the *B. coli* division produced carbon dioxide and hydrogen in approximately equal proportions whereas those of the *B. aerogenes* section produced more carbon dioxide than hydrogen. *B. coli* is termed the low ratio organism while *B. aerogenes* belongs to the high ratio group.

By the use of the hydrogen electrode, Clark and Lubs (1915 and 1917) followed the changes in the hydrogen ion concentration in various cultures of *B. coli* and *B. aerogenes*. Simple conditions were found whereby the metabolism was so controlled that the pH of cultures of one group were made to diverge widely from those of the other group. It was further found that the differences in the final hydrogen ion concentrations of cultures of *B. coli* and *B. aerogenes* correlated perfectly with their gas ratios. This principle was made use of by Clark and Lubs (1915 and 1917), and Clark (1918) in the so-called methyl red test.

Other differential tests for the separation of the coli and aerogenes divisions of the colon group have been advocated. Voges and Proskauer (1898) recommended the so-called V. P. test which depends on the formation of acetyl methyl carbinol by *B. aerogenes* but not by *B. coli*. Koser (1918) used uric acid

as the only source of nitrogen and found that *B. aerogenes* was able to utilize this compound. *B. coli* cannot use uric acid as a nitrogen source. Another test recommended by Koser (1923 and 1924) is the citrate test giving a positive growth with *B. aerogenes*. On the other hand *B. coli* fails to grow in this medium. Simmons (1926) added agar and brom-thymol blue to Koser's citrate solution and used it as a solid plating medium.

It can be seen that with the exception of Simmon's citrate agar all of the media named above are liquid media in which it is not possible to make a study of the colonies of the growing organisms. In no case can the growth of *B. coli* be studied on solid media. It was considered of great advantage to possess a solid streaking medium which would differentiate the two main subgroups of the colon group, and at the same time permit a study of the organisms growing in colonies.

Experimental

Erythrosine, methylene blue and brom-cresol-purple were the dyes selected to be incorporated in a buffered lactose agar base. The combination of erythrosine and methylene blue gives a bright metallic appearance to the colonies of *B. coli* but not to those of *B. aerogenes*. *B. coli* changes the color of the brom-cresol-purple from purple to orange while *B. aerogenes* is unable to affect the color of the indicator. The medium gives two tests in one, namely, the presence or absence of a metallic sheen and a positive or negative color change in the agar surrounding the colonies.

The medium is similar to one previously reported by the writer (Salle, 1927) but has been modified to give sharper reactions and more satisfactory results.

Erythrosine, methylene blue and brom-cresol-purple change in color from purple to orange at about pH 5.6. A medium in order to give satisfactory results must be so prepared that *B. coli* will grow and produce its maximum acidity below pH 5.6. This is achieved by the addition of buffer substances to the medium. In table 3 the results of a preliminary test with five different kinds of broth containing increasing amounts of buffer

TABLE 3

The effect of an increase in the buffer content on the final pH of the medium

CULTURE NUMBER	MEDIA									
	(1)*		(2)		(3)		(4)		(5)	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
1	4.55	4.51	4.78	4.70	4.92	4.80	5.07	4.90	5.17	5.16
2	4.62	4.60	4.80	4.72	4.97	4.78	5.02	4.92	5.14	5.07
3	4.46	4.38	4.77	4.63	4.89	4.75	4.95	4.84	5.12	5.07
4	4.33	4.29	4.56	4.41	4.68	4.55	4.82	4.65	5.02	4.87
5	4.62	4.55	4.82	4.77	4.95	4.85	5.04	4.94	5.17	5.14
6	4.60	4.56	4.92	4.85	5.00	4.87	5.14	5.00	5.26	5.17
7	4.48	4.40	4.70	4.62	4.80	4.75	4.87	4.82	5.04	4.94
8	4.43	4.45	4.75	4.68	4.85	4.80	4.92	4.89	5.06	5.11
9	4.45	4.41	4.68	4.63	4.85	4.70	4.94	4.84	5.12	5.09
10	4.53	4.50	4.84	4.75	4.94	4.95	5.09	4.97	5.19	5.16
11	4.45	4.41	4.68	4.63	4.89	4.80	4.95	4.84	5.14	5.07
12	4.33	4.33	4.62	4.46	4.72	4.62	4.78	4.60	4.90	4.90
13	4.45	4.41	4.65	4.60	4.77	4.70	4.80	4.72	5.04	4.97
14	4.58	4.53	4.80	4.72	4.89	4.82	5.04	4.92	5.17	5.11
15	4.63	4.58	4.85	4.78	5.00	4.92	5.02	4.94	5.16	5.11
16	4.50	4.46	4.68	4.67	4.80	4.72	4.87	4.77	5.04	4.97
17	4.51	4.45	4.77	4.68	4.94	4.80	5.00	4.85	5.14	5.11
18	4.63	4.63	4.82	4.77	4.99	4.90	5.09	4.94	5.21	5.17
19	4.53	4.50	4.85	4.78	4.99	4.92	5.12	5.06	5.26	5.17
20	4.36	4.31	4.58	4.50	4.70	4.63	4.78	4.72	4.97	4.90
21	4.62	4.56	4.80	4.72	4.94	4.85	5.06	4.97	5.17	5.11
22	4.62	4.55	4.85	4.75	5.02	4.78	5.09	4.90	5.21	5.16
23	4.53	4.46	4.78	4.70	4.95	4.85	5.06	4.95	5.16	5.14
24	4.45	4.43	4.63	4.58	4.84	4.75	4.92	4.82	5.07	5.11
25	4.53	4.46	4.73	4.68	4.87	4.80	4.92	4.92	5.11	5.07
26	4.46	4.43	4.68	4.63	4.84	4.77	4.92	4.85	5.11	5.06

* (1) Peptone, Difco, 5 grams; lactose, 5 grams; distilled water to 1000 cc.
(2) Peptone, Difco, 5 grams; K_2HPO_4, 1 gram; lactose, 5 grams; distilled water to 1000 cc.
(3) Peptone, Difco, 10 grams; K_2HPO_4, 1 gram; lactose, 5 grams; distilled water to 1000 cc.
(4) Peptone, Difco, 10 grams; K_2HPO_4, 2 grams; KH_2PO_4, 0.5 gram; lactose, 5 grams; distilled water to 1000 cc.
(5) Peptone, Difco, 10 grams; K_2HPO_4, 3 grams; KH_2PO_4, 0.6 gram; lactose, 5 grams; distilled water to 1000 cc.

substances are summarized. Pure cultures of *B. coli* were used. The final hydrogen ion concentrations of the cultures were determined by means of the hydrogen electrode. The tubes were incubated at 37°C. and the results recorded at the end of twenty-four and forty-eight hours. It is quite evident that as the amount of buffer is increased the final pH of the medium is correspondingly increased. These results are in harmony with the statement by Clark (1915) that the greater the buffer effect of the medium the lower the final hydrogen ion concentration attained.

TABLE 4

Final hydrogen ion concentrations of cultures of B. coli

Medium: Peptone, 10 grams; K_2HPO_4, 3 grams; KH_2PO_4, 0.6 grams; water, 1000 cc.

CULTURE NUMBER	pH IN DIFFERENT MEDIA AT DIFFERENT TIMES							
	1 per cent lactose		0.6 per cent lactose		0.5 per cent lactose		0.4 per cent lactose	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
1	4.99	4.94	5.06	4.97	5.09	4.99	5.12	5.16
2	5.06	4.97	5.07	5.07	5.17	5.07	5.26	5.19
3	4.94	4.85	4.97	4.90	4.99	4.94	5.14	5.19
4	5.04	4.90	5.09	4.92	5.22	5.02	5.60	6.29
5	4.72	4.62	4.75	4.62	4.85	4.77	4.97	4.90
6	5.02	4.94	4.99	4.94	5.04	4.95	5.12	5.04
7	5.06	4.94	5.09	4.99	5.14	4.95	5.24	5.26
8	4.99	4.92	5.04	4.90	5.17	5.04	5.38	5.56
9	5.00	4.97	5.04	4.92	5.17	5.09	5.65	6.09
10	5.06	5.04	5.14	5.00	5.21	5.16	5.61	6.27
11	4.82	4.62	5.02	4.66	5.00	4.90	5.63	6.32
12	4.84	4.62	4.97	4.65	5.04	4.77	5.29	5.33
13	4.87	4.84	4.92	4.85	5.02	4.94	5.21	5.28
14	5.02	4.97	4.99	4.97	5.14	5.00	5.22	5.22
15	4.99	4.87	5.04	4.90	5.16	5.02	5.60	6.24
16	5.07	5.04	5.09	5.06	5.19	5.07	5.56	6.00
17	4.89	4.85	4.95	4.87	5.11	4.94	5.11	5.07
18	4.90	4.89	4.94	4.90	4.97	4.95	4.97	5.12
19	4.97	4.94	5.00	4.94	5.07	4.99	5.21	5.24
20	5.07	4.95	5.11	4.97	5.21	5.00	5.44	5.63
21	5.02	4.92	5.07	4.97	5.16	4.99	5.50	5.88
22	4.85	4.85	4.94	4.90	5.04	4.99	5.14	5.11
23	4.70	4.67	4.82	4.73	4.85	4.80	4.99	4.95
24	5.02	4.92	5.07	4.92	5.21	4.99	5.56	6.10
25	4.97	4.94	4.99	4.97	5.06	4.99	5.14	5.12

For a given amount of sugar *B. coli* will form more acid than *B. aerogenes*, provided the greatest amount added is just sufficient for *B. coli* to produce its maximum hydrogen ion concentration. The amount of lactose that is just sufficient for *B. coli* to produce its maximun acidity is inadequate for *B. aerogenes* to produce its limiting hydrogen ion concentration. In table 4 are shown results obtained by inoculating lactose broth with cultures of *B. coli*, using 1, 0.6, 0.5, and 0.4 per cent lactose respectively. Formula 5, (table 3) was used. Readings were made at the end of twenty-four and forty-eight hours of incubation at 37°C. It is seen that as the amount of lactose is decreased the final hydrogen ion concentration is also decreased until a concentration is reached (0.4 per cent lactose) which is insufficient for *B. coli* to produce its characteristic limiting pH in the above medium. In order to sharply differentiate *B. coli* from *B. aerogenes* the amount of lactose added must be just sufficient for *B. coli* to produce its limiting hydrogen ion concentration. An excess may permit *B. aerogenes* to produce the same pH and the medium will be of little value. The results shown in table 3 suggested 0.5 per cent lactose as the most suitable quantity.

In table 5 are shown results with *B. aerogenes* employing the same medium and lactose concentrations as used for *B. coli* (table 3). It is seen that when results of like concentrations of lactose are compared *B. coli* cultures produce a lower pH than tubes inoculated with *B. aerogenes*. This is just what is desired as a sharp differentiation between these two organisms. The desired result is, therefore, possible.

The medium which was finally selected and which is now in use has the following composition:

Peptone, Difco	10 grams
K_2HPO_4	3 grams
KH_2PO_4	0.6 gram
Agar	20 grams
Distilled water	1000 cc.

No adjustment of the reaction is required. This is controlled by the buffer substances. The ingredients are mixed and boiled

until the agar is dissolved. The loss of water due to evaporation is restored. The agar is not filtered, but distributed directly into 250 cc. Erlenmeyer flasks, 100 cc. to each flask. When ready

TABLE 5

Final hydrogen ion concentration of cultures of B. aerogenes

Medium: Peptone, 10 grams; K_2HPO_4, 3 grams; KH_2PO_4, 0.6 gram; water, 1000 cc.

CULTURE NUMBER	pH IN DIFFERENT MEDIA AT DIFFERENT TIMES							
	1 per cent lactose		0.6 per cent lactose		0.5 per cent lactose		0.4 per cent lactose	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
1	5.68	5.26	5.61	5.88	5.73	6.54	5.88	6.70
2	5.61	5.24		6.32	5.73	6.42	6.04	6.71
3	5.41	5.34	5.63	6.31	5.73	6.42	6.29	6.85
4	5.55	5.24	5.65	6.15	5.78	6.59	5.82	6.58
5	5.14	5.78	5.77	6.58	6.19	6.91	6.27	6.97
6	5.12	5.66	5.73	6.53	5.83	6.75	6.24	6.88
7	5.16	5.51	5.97	6.81	6.00	6.76	6.48	6.95
8	5.70	5.22	5.60	5.93	5.65	6.41	5.77	6.36
9	5.77	5.39	5.44	5.90	5.80	6.42	5.90	6.66
10	5.58	5.36	5.63	6.19	5.75	6.32	6.19	6.91
11	5.48	5.14	5.51	5.95	5.66	6.63	5.82	6.68
12	5.12	5.75	5.88	6.49	6.27	6.76	6.48	6.85
13	5.58	5.39	5.63	6.15	5.70	6.29	6.19	6.81
14	5.12	5.38	5.85	6.58	5.71	6.46	6.51	6.93
15	5.31	5.44	5.56	6.04	5.61	6.31	6.19	6.75
16	5.28	5.11	5.60	6.09	5.82	6.49	6.22	6.91
17	5.75	5.36	5.66	6.15	5.71	6.80	5.93	6.78
18	5.17	5.51	5.73	6.54	6.22	6.64	6.44	7.05
19	5.16	5.36	5.83	6.49	6.14	6.71	6.39	6.83
20	5.50	5.22	5.56	6.10	5.58	6.26	5.77	6.46
21	5.40	5.06	5.58	6.15	5.68	6.31	5.87	6.61
22	5.48	5.38	5.80	6.54	6.15	6.68	6.22	6.86
23	5.68	5.43	5.50	6.20	5.78	6.49	6.00	6.80
24	5.34	5.17	5.56	6.24	5.73	6.58	6.36	7.05
25	5.53	5.46	5.73	6.46	5.99	6.63		
26	5.43	4.99	5.61	6.09	5.77	6.37	6.12	6.59

to use, 0.5 gram lactose is added to each flask and the agar melted in an Arnold sterilizer or in boiling water. After the agar is melted the following dyes are added to the contents of each flask: erythrosine (2 per cent aqueous), 2 cc.; methylene blue (1 per cent aqueous) 0.2 cc.; and brom cresol purple (1 per cent

alcoholic) 2 cc. The agar is rotated in the flask to obtain an uniform mixture and then poured into sterile petri dishes.

Preparation of dye solutions

Methylene blue, 1 per cent. Dissolve 1 gram of methylene blue in 100 cc. of distilled water. This may be conveniently carried out by placing the dye in a glass stoppered bottle, adding the distilled water and shaking vigorously until completely dissolved.

Brom cresol purple, 1 per cent. Place 1 gram of the indicator in a mortar, add 25 cc. of 95 per cent alcohol and triturate until completely dissolved. Then add sufficient distilled water to make 100 cc.

Erythrosine, 2 per cent. Place 2 grams of the dye in a mortar and add about 50 cc. of distilled water. Then add 5 per cent KOH solution, drop by drop, until the dye is completely dissolved. Triturate well after each addition in order to avoid adding a large excess of alkali. Finally make up the volume to 100 cc. with distilled water.

At the time this work was started it was not appreciated that different brands of dyes or even different lots of the same manufacturer's product could vary so greatly in their physical and chemical properties. It makes very little difference which brands of methylene blue and brom cresol purple are used but the difficulty lies with the erythrosine. With the exception of a small quantity of pre-war erythrosine on hand at the time no other brand has been found satisfactory. Work was, accordingly, started on the preparation of a satisfactory dye with the result that a product has been developed that is far superior to any of those tested and which has given very excellent results.

Preparation of erythrosine

A. Fluorescein U. S. P. sodium salt (National Aniline and Chemical Company)	20 grams
Distilled water	120 cc.

Dissolve the fluorescein in the distilled water contained in a 400 cc. beaker.

B. Iodine U. S. P.	40 grams
Potassium iodide, U. S. P.	40 grams
Distilled water	340 cc.

Grind the iodine and the potassium iodide together in a mortar until finely divided. Rinse out the mortar with small portions of distilled water until the contents is all dissolved. Collect the solution into a liter beaker. Heat the mixture to 75°C. to dissolve all of the iodine. Then add to it, with constant stirring, the fluorescein solution (A). The erythrosine immediately precipitates out. Allow to stand for five minutes, again heat to 75°C. and pour out the contents into an enameled pan containing two liters of distilled water. Bring the contents of the pan to a boil, remove from the fire and allow to stand for five minutes for the precipitate of erythrosine to settle out. The scum that forms on the surface may be broken up and made to settle out by vigorously stirring while the mixture is boiling. Carefully pour off the supernatant liquid, containing free iodine, in order to not disturb the precipitate. Add two more liters of distilled water to the precipitate and again bring to a boil. After allowing to stand for five minutes carefully pour off the supernatant fluid. Repeat the operation for the third time but use only one liter of distilled water. Add 125 cc. of 5 per cent KOH solution to dissolve the precipitate of erythrosine. Pass the clear solution through a 5-inch Buchner funnel with the aid of suction. Dilute the filtrate with sufficient distilled water to make about one liter. Bring to a boil and add about 20 cc. of concentrated HCl with constant stirring to precipitate the erythrosine. Allow to stand for five minutes. Pour off most of the supernatant liquid and collect the precipitate on a 5-inch Buchner funnel using suction. Rinse out the pan with several portions of distilled water, dry the precipitate well with suction, finally drying thoroughly in the incubator or in a warm place. When dry, pulverize in a mortar and preserve in a well stoppered bottle.

On this medium the colon organisms produce brilliant metallic

colonies, slightly raised, with the surfaces usually flat or slightly concave. The surfaces are rarely convex. The colonies remain confined and show little tendency toward confluence. The agar surrounding the colonies will be changed in color from a purple to orange tinge. *B. aerogenes* strains rarely, if at all, produce metallic colonies and then only in the central portions. The

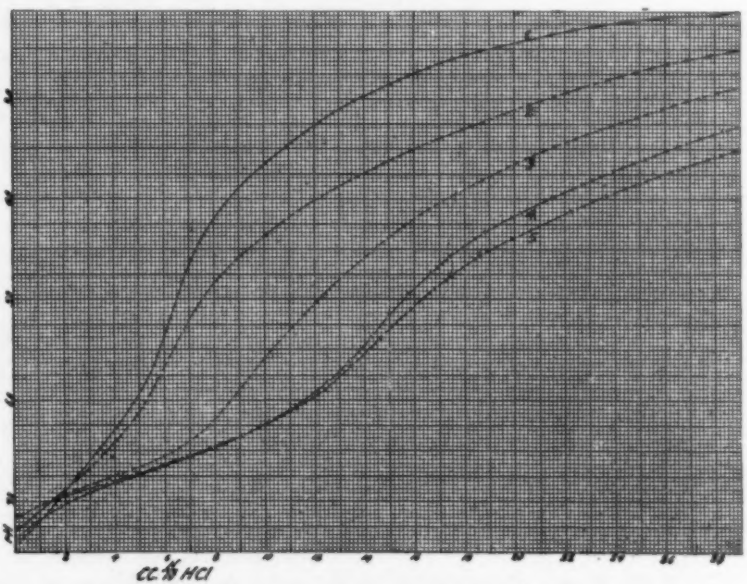

FIG. 1. TITRATION CURVES OF MEDIA GIVEN IN TABLE 3

pH readings by hydrogen electrode, and titration figures in cubic centimeters of N/10 HCl for 100 cc. of medium are used.

colonies are raised, convex, very moist and show great tendency to run together. There is no change in the color of the agar.

In figure 1 are shown titration curves of the five media given in table 3. One hundred cubic centimeters of the media were titrated with N/10 HCl and the pH readings followed by means of the hydrogen electrode. Titration curves are very useful in that they tell us at what pH range the media exert their maximum

buffer effect. They also help others to duplicate results. For instance, it makes no difference what brand of peptone is used as long as the titration curves are the same. Taking up the curve for medium 5, table 3, it is evident that it requires about one-half as much acid to change the pH of the medium from 6 to 5 as to change the pH from 7 to 6. *B. aerogenes* produces its maximum acidity (using 0.5 per cent lactose) at about pH 6.0; *B. coli* has its limiting hydrogen ion concentration at about pH 5.0. Between pH 6.0 and 5.0 a relatively large change is obtained for a given amount of acid as compared with the pH range between 7.0 and 6.0. This is just what is desired, since such a medium will afford a sharp differentiation of *B. coli* and *B. aerogenes*.

TABLE 6

Results obtained by streaking pure cultures of B. coli and B. aerogenes on the differential medium

ORGANISM	STRAINS	REACTION ON AGAR		CORRELATION
		Metallic colony	Color	
				per cent
B. coli	180	+	Orange	100
B. aerogenes	50	−	Purple	100

Results

In table 6 are shown results obtained by streaking on the surface of the medium 180 strains of *B. coli* and 50 strains of *B. aerogenes*. The results show perfect correlation.

The solid medium here described incorporates two tests in one operation, namely, the presence or absence of a metallic sheen to the colonies and a positive or negative color change in the agar. In water examinations when speed is very essential, the agar dye preparation will shorten the period to complete an analysis by twenty-four hours.

Instead of fishing metallic colonies from an Endo or an E. M. B. plate and making tests for the presence of fecal *B. coli* (which requires twenty-four hours more time) the results may now be

obtained on the plate. In laboratories where it is the custom to carry the test only to the partially confirmed stage, more satisfactory conclusions may now be drawn with the aid of this medium, since two tests are better than one.

Results of water examinations

More than 100 water examinations were carried out using Standard Methods broth and crystal violet broth media. Five tubes of lactose broth and crystal violet broth each containing 10 cc. of water sample were used for each sample of water examined. Readings were made at the end of forty-eight hours. The results are given in table 7.

TABLE 7

Results of water examinations using standard broth and crystal violet broth

		NUMBER OF TUBES
Samples examined	101	
Perfect correlation between standard and crystal violet broth	62	
Samples giving higher results in standard broth	20	33
Samples giving higher results in crystal violet broth	11	18
Samples in which standard broth tubes did not confirm for members of the coli-aerogenes group	18	33
Samples in which crystal violet broth tubes did not confirm for members of the coli-aerogenes group	1	1

The results tabulated in table 7 bring out the superiority of crystal violet broth over Standard lactose broth. Of 101 samples analyzed 62 showed perfect correlation. Twenty samples in standard broth gave 33 more positive tubes than crystal violet broth. However, on confirmation 18 of the above samples including 33 tubes gave either no growth or negative results for members of the coli-aerogenes group. Eleven samples including 18 tubes gave higher results in crystal violet broth all of which confirmed, with the exception of one tube.

The results clearly show that gas formation in crystal violet broth in practically 100 per cent of the tubes is due to the presence of members of the coli-aerogenes group. Other organisms are

eliminated by the dye in almost all cases, giving sharper and more clear cut results on the confirmatory plates. A considerable saving in time and materials is thus accomplished by incorporating crystal violet in the medium.

Of the 20 samples mentioned, twenty-four hours additional time was required to complete an analysis. This time would have been saved by the use of crystal violet broth. Also the majority of the 20 samples were obtained from chlorinated supplies where the treatment did not destroy anaerobic and aerobic spore formers capable of fermenting lactose with the production of acid and gas. None of these organisms produced positive results in crystal violet broth.

The dye broth will show its superiority particularly with the chlorinated samples which tend to give a large percentage of false tests in Standard broth. Of the 101 samples examined only a small number were from chlorinated supplies. If the number of chlorinated samples had been greater the results would have been even more striking.

DISCUSSION

A system for the bacteriological examination of water is proposed which it is believed will prove superior to any now in use.

Comparative tests of brilliant green and crystal violet have shown the superiority of the latter for water examinations. Brilliant green has enjoyed widespread popularity for water examinations but it is not clear just why this dye has been selected in preference to crystal violet.

A crystal violet broth medium is described which in 101 samples examined has been shown to give practically 100 per cent results for members of the coli-aerogenes group. With the use of such broth much time will be saved due to the elimination of those organisms capable of giving acid and gas in standard lactose broth but which do not confirm for the members of the colon group.

An agar dye medium for the confirmatory test is described which incorporates two tests in one operation. In reality, results on this plate medium are equivalent to a partially confirmed

and a confirmed test for the presence of *B. coli*. The *B. coli* organisms produce bright metallic colonies while *B. aerogenes* colonies do not produce a metallic appearance, except in very rare cases. The amount of buffer and lactose in the medium are so controlled that brom-cresol-purple will change in color from purple to orange by the growth of *B. coli* but not by *B. aerogenes*. In those rare instances in which colonies of *B. aerogenes* give a metallic lustre the absence of a change in the color of the agar will distinguish them from *B. coli*.

Comparative tests on 101 samples, using standard broth and crystal violet broth have shown the superiority of the latter in water analysis. Much time and materials will be saved due to the elimination of these organisms giving false tests in water examinations. Also, it is no longer necessary to make confirmatory tests on colonies picked from the plate. Two tests are now incorporated in one operation thereby shortening the period of a complete water analysis by twenty-four hours.

SUMMARY

1. Comparative tests using the five organisms, *B. coli*, *B. aerogenes*, *B. subtilis*, *Staphylococcus aureus* and *Staphylococcus albus* have shown the superiority of crystal violet over brilliant green as a bacteriostatic agent.

2. A crystal violet broth medium for the presumptive test is described: containing peptone (Difco), 15 grams; K_2HPO_4, 15 grams; KH_2PO_4, 3 grams; lactose, 15 grams; crystal violet (1:5000 solution), 21.5 cc.; distilled water, to make 2000 cc.

3. Production of acid and gas in this medium in practically 100 per cent of the samples examined is due to members of the coli-aerogenes group.

4. An agar-dye differential medium is described: containing peptone, (Difco), 10 grams; K_2HPO_4, 3 grams; KH_2PO_4, 0.6 gram; lactose, 5 grams; agar, 20 grams; distilled water, 1000 cc.; erythrosine (2 per cent aqueous), 20 cc.; methylene blue (1 per cent aqueous), 20 cc. Brom cresol purple (1 per cent alcoholic) 2 cc.

5. Results on this medium are equivalent to a partially con-

firmed and a confirmed test. In other words, two tests are obtained in one operation, thus shortening the period to complete an analysis by twenty-four hours. *B. coli* and *B. aerogenes* are sharply differentiated because of distinct differences in their carbohydrate metabolism.

6. Results of water examinations are detailed showing the superiority of the method over that given in "Standard Methods." The method gives, not only more clear cut results due to elimination of interfering non-colon organisms, but also a saving of at least twenty-four hours in the time necessary to complete an examination.

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MICROCOCCUS NIGER, A NEW PIGMENT-FORMING ANAEROBIC COCCUS RECOVERED FROM URINE IN A CASE OF GENERAL ARTERIOSCLEROSIS

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The obligately anaerobic cocci are so difficult to isolate in pure culture and to maintain after isolation that they still remain one of the least studied groups of all bacteria. There is no question, however, that they are quite common in nature and it is only the inadequacy of our technic and our general lack of knowledge of them that causes us frequently to overlook them in mixed cultures, where, if anaerobic bacilli are present, the anaerobic cocci are almost certain to be lost in the process of isolation by heating and other selective methods particularly designed for sporulating bacteria.

In some cases, however, where the anaerobic bacilli are absent and none of the aerobic organisms recovered produces gas as the original mixed culture does, one may suspect the presence of non-sporulating anaerobes, and frequently, although of course by no means always, these prove to be anaerobic cocci, for many of the anaerobic cocci, unlike the aerobic cocci, produce gas in suitable media. *M. gazogenes* (Lewkowicz) is one of the commonest, particularly in the mouth (Hall and Howitt, 1925) and it frequently occurs in the naso-pharynx (Branham, S. E., 1927, 1928; Noble and Brainard, 1928) and in the genito-urinary system (Schmid and Kamniker, 1926).

In the present instance, a specimen of catheterized urine was examined from an elderly psychotic woman suffering from general arteriosclerosis and ergot poisoning, in whom gaseous gangrene caused by *Bacillus Welchii*, *Micrococcus aureus* and *Streptococcus*

ignavus infection of one hand following an amputation required by endarteritis obliterans had been averted by the timely use of antigangrene serum and thorough drainage. The patient died a few weeks later from general sepsis but no autopsy could be secured.

The urine was slightly turbid. Upon centrifugalization no pus cells were seen but there were numerous Gram positive diplococci, streptococci, and a few long Gram negative filaments.

Primary cultures were made upon lactose-eosin-methylene blue plates, blood agar plates, and in milk (constricted tubes) and deep brain medium. No coliform colonies appeared upon any of the plates, and *B. coli*, which is so common in urinary infections, was not isolated. *Streptococcus fecalis* and *Micrococcus albus* were isolated from the blood agar plate. The milk culture was coagulated after twenty-four hours and showed gas production at forty-eight hours but no stormy fermentation such as might suggest the possible presence of *B. Welchii*. The deep brain culture also produced gas but the microscopic examination showed only Gram-positive diplococci, streptococci and Gram-negative rods. Being unable to isolate any Gram-negative rod by aerobic means, I assumed that an anaerobic organism of this type might be responsible for the gas production in the primary aero-anaerobic cultures, and the usual deep agar dilution cultures in Burri tubes were prepared for its isolation (Hall, 1929). Numerous well separated colonies were fished from these and several of them showed gas production in brain medium, but, upon checking, all were found to consist of the aerobic cocci above mentioned mixed with some unknown anaerobic gas former. The Gram negative filaments and rods never appeared, however, in any of the isolation cultures and were no doubt lost.

The daily manipulations designed to isolate the gas former lasted more than six weeks and this fact is mentioned only to indicate the very great difficulty of isolation and to emphasize the point that if some other gas former had been isolated the present organism would perhaps have been entirely overlooked. Early in this period my attention was drawn to a few small densely black colonies appearing in certain deep agar cultures that had been

incubated for about five days, which is longer than we usually incubate them before picking colonies. These suggested the possible presence of the *Bacterium melaninogenicum* of Oliver and Wherry (1921) and of Burdon (1928) although no reference to gas production is made by these authors. Finally, after many failures and by repeated picking of black colonies from deep agar to brain medium, checking those showing gas for aerobic contamination (always *M. albus* or *Strep. fecalis*) and again making deep agar dilution cultures, three colonies were picked which gave gas in brain medium and no aerobic growth on plain agar slants. All of them proved to be pure cultures of the organism here designated "*Micrococcus niger*," species *novum*.

MORPHOLOGY AND TINCTORIAL REACTIONS

M. niger is a small Gram-positive coccus measuring about 0.6μ in diameter and forming irregular masses resembling staphylococci. Diplococci occurred occasionally but no true chains were formed.

CULTURAL PROPERTIES

M. niger is obligately anaerobic. Numerous check tests upon plain and blood agar slants failed to show any aerobic growth. The usual methods of securing anaerobiosis are quite adequate, however, for its culture. Growth was slow upon all media at 37°, requiring at least two to four days before becoming visible. At room temperature (about 25° to 30°C.) in controlled experiments with deep agar about seven days were required.

Deep 1 per cent meat infusion agar with 2 per cent peptone, pH 7.0. Colonies appeared first at 37°C. after about two days incubation. If thickly seeded, small bubbles of gas sometimes appeared in the agar. The colonies were at first colorless, but about the fourth day began to show a brownish color, turning to dull black about the fifth or sixth day. The pigment was strictly localized in the colonies; there was never any discoloration of the medium suggesting diffusion or solubility. Even though well isolated the colonies rarely exceeded 0.5 mm. in diameter and were irregularly globular, smooth and dense. Owing to their strictly anaerobic re-

quirements and slow growth, no colonies were usually found nearer than 3 cm. to the surface. Curiously, as the medium dried out after a few weeks, admitting air alongside the agar plug, the colonies so exposed faded to a dull gray.

The addition of several cubic centimeters of ascitic fluid to each tube of deep agar apparently made no difference in the rapidity or character of growth.

Brain medium. Single colonies picked pure from some deep agar into brain medium showed turbidity first only after four or five days incubation at 37°C., and gas production uniformly after the sixth day. Heavily seeded cultures sometimes showed gas after one day's incubation. I tried but was unable to prove acceleration of gas production in brain medium due to synergy or symbiosis with *Streptococcus fecalis.* Pure cultures showed only a slight blackening of deep brain containing iron wire after prolonged incubation, due presumably to the production of sulfuretted hydrogen. The fact that the discoloration in this medium is not marked suggests that the pigment formed in agar is probably not directly connected with the production of sulfuretted hydrogen and the formation of iron sulfide as in the case of the putrefactive anaerobic bacilli.

Blood agar slants under alkaline pyrogallol. No growth visible to the naked eye appeared until the fourth or fifth day. Then minute black colonies arose like tiny black pearls, round, smooth and glistening, reaching next day their maximal size of 0.5 mm. They were non-hemolytic even after two weeks.

Loeffler's coagulated blood serum under alkaline pyrogallol. I was unable to note any growth until the eighth day when minute brown colonies appeared discretely over the white surface of the serum. The largest of these later reached a maximal size of 0.5 mm. but did not become as black as in agar. There was no liquefaction of the serum in five weeks.

Gelatin. In the constricted tube the gelatin remained clear throughout the entire period of observation, but a dark sediment became noticeable on the fifth day, gradually becoming more and more intensely black. There was no liquefaction after twelve days incubation at 37°C. as shown by the hardening of the gelatin in the ice box. Identical results were obtained under vaspar seal.

Milk. There was no evidence of any growth or change on ten days incubation at 37°C., either in the constricted tube or under vaspar seal.

Fermentation tests. Glucose, levulose, galactose, maltose, glycerol and xylose, tested in constricted tubes with marble seals showed turbidity first on the second day, and a trace of gas on the third day. A black sediment collected in the bottom of the tubes. There was no production of acid; the cultures uniformly became more alkaline. It seems probable that no carbohydrates are fermented.

NON-PATHOGENICITY FOR ANIMALS

M. niger is non-pathogenic for guinea pigs inoculated subcutaneously and for rabbits inoculated intravenously. I tried 2 cc. of a four-day-old glucose broth culture in a 200-gram guinea pig without any result. I also inoculated a 2500-gram rabbit intravenously with 5 cc. of a five-day glucose broth culture without effect.

DISCUSSION

There are only a few references in the literature to brown or black pigment forming cocci. One of the earliest was that of Sternberg (1900) who described an obligately anaerobic long chained streptococcus from sputum in a case of human pulmonary actinomycosis, which was pathogenic for rabbits, and formed dark brown or black colonies upon sugar agar plates.

In 1903, Löw having observed a dark colored microbic growth upon the surface of a solution of chinic acid neutralized with chalk, Emmerling and Abderhalden (1903) repeated the conditions and isolated a coccus, which they called "*Micrococcus chinicus*" and which reproduced the pigment upon chinic acid neutralized with chalk by the oxidation of calcium chinate to protocatechuic acid; under anaerobic conditions naturally no oxidation occurs and the colonies were colorless. This phenomenon was subsequently studied by Beijerinck (1911) who showed that various bacteria, notably *Bacillus prodigiosus, Aerobacter aerogenes, Pseudomonas aromatica, P. fluorescens non-liquefaciens, P.*

fluorescens liquefaciens, *P. pyocyaneus*, and a coccus, closely related to *M. chinicus*, but according to Beijerinck distinct, and named by him "*M. calco-aceticus*," all oxidize chinic to protocatechetic acid, as it is now called. Beijerinck further showed that brown or black colored compounds may be formed by other aerobic bacteria by other oxidations, for example, quercite to pyrogallic acid and its colored compounds in the presence of alkali and oxygen by *Pseudomonas aromatica*, tyrosine to homogenetisic acid and melanin by *Microspira tyrosinatica*, and possibly gelatin or peptone to chinon by *Acetobacter melanogenum*. It seems obvious, however, that none of these oxidations can account for the production of pigment by an obligately anaerobic organism such as *M. niger*.

In 1907, Gräf and Wittenben isolated two species of anaerobic streptococcus forming brown or black colonies in deep agar. Their Streptococcus "Sch" (Schwartzenbek), from an actinomycoid swelling in the neck of a peasant, formed long chains of 30 to 50 elements and liquified gelatin; their Streptococcus "K" (Kiel) from a brain abscess, coagulated and peptonized milk, so that *M. niger* is easily distinguished from these.

In 1910, Schottmüller recovered a long chained obligately anaerobic streptococcus from puerperal infections, which he named *S. putridus*. The same germ was again recovered by Marwedel and Wehrsig (1915) from war wounds. According to Weinberg and Seguin (1917) it turns blood broth black and produces a putrid odor. Schwartz and Dieckmann (1926) (1927) suggested that the blackening of blood cultures by *S. putridus* might be due to melanin, but I believe there is no proof for this. Numerous studies (Abderhalden, 1909; Kastle, 1909; Gortner, 1911) have shown that melanin is formed by the oxidation of tyrosin to homogentisic acid by tyrosinase, and while some aerobic bacteria apparently produce melanin in this manner, according to Gessard (1898, 1901), Lehman (1902), Lehman and Sano (1908), and Beijerinck (1911), under aerobic conditions, no pigment is formed by any of these organisms when they are grown anaerobically in the presence of tyrosine, and it is impossible to explain the pigment production of an obligate anaerobe in this manner.

There is no question of the separate identity of *S. putridus* and *M. niger;* the long chains and putrid odor of the former clearly distinguish it.

On three different occasions, Gilmer and Moody (1914) observed black pigment forming organisms in old anaerobic cultures from alveolar abscesses but did not succeed in isolating them. I have had similar experiences in dealing with cultures from the mouth; we do not know whether these are cocci or not.

Castellani and Chalmers (1919) placed the black pigment forming cocci in a separate genus, "Nigrococcus," with *Nigrococcus nigrescens* (Castellani 1911) as a type. Castellani (1926) has since described *Micrococcus* (*Nigrococcus*) *nigrescens* in detail. The relation of this organism to air was not definitely stated but the impression was left that it is aerobic. Castellani regards it as an important symbiont (or better synergist?) with *Nocardia tenuis*, in the etiology of Trichomycosis nigra.

The possession of so striking a character as the production of black pigment makes it unnecessary here to discuss the other anaerobic cocci that do not have it. For more general reviews of these organisms the interested reader may refer to the works of Jungano and Distaso (1910), Prevot (1925), Ford (1927), Weinberg and Ginsbourg (1927), Thomson and Thomson (1927), and Taylor (1929).

As to the nature of the pigment in *M. niger*, I have been unable as yet to cultivate the organism in sufficient quantity to make any determination of its character, but it is permissible, *a priori*, from its strict localization within the colonies, to suggest that it will be found to be insoluble in aqueous solutions, and from the fact of its production under anaerobic conditions that the mechanism of its elaboration differs strikingly from those chromogenic processes for the production of black pigments by aerobic bacteria, where oxidation plays so important a rôle. Furthermore the fading of the anaerobic colonies on exposure to the air suggests that studies of this pigment will be complicated by the necessity of keeping it protected from the atmosphere.

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THE EFFECT OF THE OXIDATION-REDUCTION CHARACTER OF THE MEDIUM ON THE GROWTH OF AN AEROBIC FORM OF BACTERIA[1]

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Studies on the oxidation-reduction potentials of bacterial systems have proceeded along two major lines: first, the effect of the oxidation-reduction character of the medium on the growth and activities of the bacteria; and second, the effect of the bacteria on the potentials of the medium.

The material dealing with the second phase has been reviewed by Thornton and Hastings (1929), Harvey (1929), and Coulter and Isaacs (1929). The work on the first line the effect of the potentials on the bacteria, has been confined largely to the anaerobic organisms because it is with these forms that this factor is conspicuously effective. That anaerobic bacteria can be grown in a medium exposed to atmospheric oxygen provided there is an appropriate adjustment of the oxidation-reduction character of the medium, has been shown by Berthelot (1924), Hosoya (1925), and Quastel and Stephenson (1926), and their findings have added materially to our understanding of the physiology of these organisms.

The aerobic forms grow in the presence of free atmospheric oxygen and thus but little attention has been paid to the importance of the oxidation-reduction potentials on their activities. The results of the present study deal primarily with the influence of the oxidation-reduction character of the substratum on the growth and behavior toward atmospheric oxygen of an aerobic

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form, Rhizobium, which is the causative agent of the root nodules of the Leguminosae.

THE RELATION OF THE OXIDATION-REDUCTION CHARACTER OF THE MEDIUM TO THE BEHAVIOR OF RHIZOBIUM TOWARD ATMOSPHERIC OXYGEN

The results of many tests indicate that the behavior of the nodule organism toward atmospheric oxygen depends to a large extent upon the oxidation-reduction character of the medium. For example, agar shake cultures in media of varying oxidation-reduction character show decided differences in the depth of the zone of growth. For such studies, tubes of the melted agar medium are thickly seeded with the organisms, and observations made at various intervals on the growth zones established by the bacteria. Eisenberg (1918) working with certain aerophilic spore formers and the cholera vibrio, observed that these organisms developed distinct zones of growth below the surface of glucose agar in shake cultures and attributed this behavior to an oxygen relationship. Aubel and Aubertin (1927) used a similar method in studying certain anaerobic and facultative bacteria, and endeavored to measure the potentials of the media at which the growth zones appeared by the use of oxidation-reduction indicators. Snieszko (1928) observed that the zonation of the nodule bacteria in liquid media was influenced by the hydrogen ion concentration.

The growth zones established by the nodule bacteria appeared as definite lines either at, or under, the surface depending upon the character of the medium. In so much as the organisms were seeded uniformly throughout the tubes and fixed at their respective places in the solid medium, it seems reasonable to assume that the definite growth zones established, represented regions in which the organisms found optimum conditions with respect to oxygen.

The following four media of differing oxidation-reduction character were used.

Basic mineral salts agar

K_2HPO_4	0.5 grams
$MgSO_4 \cdot 7H_2O$	0.2 grams
NaCl	0.1 grams
Agar	15.0 grams
Distilled H_2O	1000 cc.

(A) Mannitol nitrate mineral salts. One per cent of mannitol and 0.1 per cent of potassium nitrate were added to the basic mineral salts agar.

(B) Ferric ammonium citrate mineral salts. One per cent of ferric ammonium citrate was added to the basic formula.

TABLE 1

Depth of growth levels established by certain root nodule bacteria in agar shake cultures

ORGANISMS	DEPTH BELOW SURFACE			
	Mannitol nitrate medium	Ferric ammonium citrate medium	Yeast extract mannitol medium	Yeast extract glucose medium
	mm.	mm.	mm.	
Alfalfa 100	7	4	At the surface	At the surface
Clover 202	6	4	At the surface	At the surface
Pea 310	5	3	At the surface	At the surface
Bean 403	6	3	At the surface	At the surface
Soybean 504	13	8	4	At the surface
Cowpea 600	12	6	3	At the surface
Lupine 807	12	8	2	At the surface

(C) Yeast-extract mannitol mineral salts. Differs from (A) in that 100 cc. of yeast extract were substituted for the potassium nitrate.

(D) Yeast-extract glucose mineral salts. Differs from (C) in that glucose was substituted for the mannitol.

Modifications of these media were made by the addition of oxidizing or reducing substances (potassium permanganate and cysteine respectively) to increase or decrease the oxidation-reduction intensities. All of the media were adjusted to pH 6.8 when the experiments were initiated, with the exception of the ferric ammonium citrate medium.

From the data in table 1 it is seen that the level of the zone of growth of any of the organisms used in this study is determined to a great degree by the potential of the substrate; moreover, the level of this zone differs with the various organisms. The four media are arranged in table 1 in the order of their oxidation-reduction intensities, with the mannitol nitrate representing the most oxidizing and the yeast-extract glucose, the most reducing medium.

In the mannitol nitrate medium each of the organisms established growth zones well under the surface (5 to 13 mm.). This medium contained 0.1 per cent of potassium nitrate which rendered it rather oxidizing in character. A picture similar to that in the mannitol nitrate was obtained in the shake cultures of the ferric ammonium citrate medium. The growth zones of all the organisms tested appeared under the surface (3 to 8 mm.) of the medium, but at levels somewhat higher than in the first case.

In the yeast-extract mannitol medium, the growth levels appeared uniformly higher in the tubes than with either the mannitol nitrate or ferric ammonium citrate medium. The first four organisms established growth levels at the surface only. The other three appeared slightly under the surface. The substitution of the yeast water, which is reducing in character, for the potassium nitrate was the only difference between this medium and the mannitol nitrate one, in which all of the organisms grew well under the surface.

The yeast-extract glucose medium differs from the one just discussed in that glucose, which is strongly reducing, has replaced the mannitol. The growth zones of all organisms appeared at the surface only in this medium.

In general, it appears that, as the media are made more reducing in character, the growth zones appear correspondingly nearer to, or at, the surface of the medium. This conclusion was confirmed further by the addition of cysteine to the media in which the zones normally appeared below the surface. Upon the addition of approximately 0.1 per cent cysteine, the growth zones were established only at the surface of these media. As indicated by the reduced state of methylene blue, this amount of cysteine

was sufficient to maintain anaerobic conditions for some time throughout the tube except at the very surface.

After incubation for one month, a second growth zone appeared some 8 mm. under the first in the cultures of the soybean nodule organism in mannitol nitrate agar. Burnett, (1927) working with Enterococcus 775, reported a similar phenomenon when this organism was subjected to the influence of cyanide. Eisenberg (1918) also noted that certain strains of a cholera vibrio and many aerophilic spore formers in shake cultures of glucose agar often established more than one growth level, frequently evenly spaced one below the other, with the upper zone appearing first. Nowak (1908) has suggested that the appearance of two growth levels with *B. abortus* indicates that the organism possesses two optima of oxygen tension.

In the case of the soybean organisms, however, the two zones of growth do not seem to indicate the presence of two oxygen optima since the two levels were established at intervals of approximately one month, with the second zone appearing under the first. The first level was established, it is believed, at a point of optimum oxygen pressure for the organism at that time. The release of carbon dioxide and the diffusion of other metabolic by-products into the medium prevented the organisms in other regions from initiating growth. After the organisms in the first zone had run their physiological course, i.e., stopped respiring, and the medium had evaporated somewhat, oxygen again diffused in and a second optimum appeared in a region just below the toxic region established by the first—hence the second zone.

Growth in highly oxidized media

Another experiment was set up in which the medium, (yeast-extract glucose nitrate) was treated with varying amounts of potassium permanganate (0.001 to 0.04 per cent) to intensify its oxidative character, and inoculated with the clover nodule organism. This medium was a modification of the previously described yeast-extract glucose medium—D. The yeast extract was reduced to 20 cc. per liter, glucose to 4 grams, and 0.5 gram of potassium nitrate added. The hope was to attain an oxida-

tion-reduction potential at which these aerobic bacteria could derive their energy by the oxidation of the glucose and simultaneous reduction of the nitrate or permanganate and hence be grown in the absence of free atmospheric oxygen.

The potassium permanganate was sterilized separately in solution, and added to the medium in proper concentrations. Before inoculation and after the addition of the permanganate to the medium, the tubes were placed for fifteen to thirty minutes in a water bath at 50°C. It was necessary to allow the reaction between the permanganate and the medium to come to equilibrium before inoculating in order to avoid killing the organisms. Heavy inocula of the organisms, five drops of a rich suspension, were used and uniformly suspended in the medium. The tubes were incubated at room temperature and observations made on the growth levels established.

$KMnO_4$ ADDED	DEPTH OF GROWTH ZONES
per cent	
None	Surface only
0.001	Surface only
0.002	4 mm.
0.005	6 mm.
0.015	10 mm., some diffusion
0.02	Diffused growth below 10 mm.
0.04	No growth

With the addition of increasing amounts of potassium permanganate to the medium, there was a corresponding lowering of the growth levels in the medium. When the medium was oxidized to a level just short of inhibition of growth, there was a tendency for the organisms to grow throughout the medium. The behavior of these aerobic organisms in the highly oxidized media was similar to that of facultative anaerobes in nutrient agar. Whether or not these organisms, growing in the deeper layers of the medium were able to develop at a much lower oxygen tension by making use of some combined oxygen; or whether the effect of the potassium permanganate permitted the atmospheric oxygen to penetrate more deeply into the medium, remains a

question. This phenomenon, however, suggested the possibility of growing these organisms under anaerobic conditions.

A similar series of tubes was set up and incubated both under a vaseline seal and in anaerobic chambers (alkaline pyrogallol). Slight evidence of cell proliferation in the highly oxidized media was noted under the anaerobic conditions but in no case was good growth secured. In view of the slight evidences of growth secured in these tubes, it is believed that if the proper combination of the ingredients of the medium were obtained and the oxidation-reduction potential properly adjusted, these organisms might be able to use combined oxygen and grow in the absence of atmospheric oxygen.

THE EFFECT OF OXIDIZING AND REDUCING SUBSTANCES ON THE GROWTH OF THE CLOVER ROOT NODULE ORGANISM IN PLATE CULTURES

In Petri plate cultures, the layer of the substrate is usually thin and exposed to atmospheric oxygen. Certain strains of the root nodule organisms establish growth levels in shake cultures (test tubes) at a depth greater than is commonly found in Petri plate cultures. These deep zones of growth are especially characteristic of highly oxidized media. The question which naturally arose was, what effect would oxidizing and reducing substances have upon the growth of these aerobic organisms in thin substrates characteristic of Petri plate cultures. To test this idea restricted areas in Petri cultures were activated by blocks of plain agar adjusted to the proper pH, in which either oxidizing or reducing compounds had been incorporated.

Five oxidizing compounds were used in the blocks: (1) potassium permanganate, (2) potassium nitrate, (3) potassium chlorate, (4) ferric ammonium citrate, and (5) hydrogen peroxide. The reducing materials used were: (1) cysteine, (2) ferrous ammonium sulfate and (3) living potato blocks.

The agar blocks were prepared in the following manner. The oxidizing or reducing compounds in known concentrations were sterilized. A 2 per cent plain agar solution, adjusted to pH 6.8, was sterilized, and cooled to 45°C. The proper amounts of the

oxidizing or reducing compounds were added and the agar poured into Petri plates in 4 to 5 mm. layers. Subsequently, small pieces of this layer were cut out and introduced aseptically into other Petri plates preparatory to seeding the clover root nodule organisms around them in the media to be tested. The organisms were homogeneously suspended in the media before pouring the same around the oxidizing and reducing agar blocks.

A few exceptions necessarily were made to the procedure just described. The ferrous ammonium sulfate and hydrogen peroxide were not sterilized but introduced aseptically. The sterile potato blocks were cut from the heart of a potato and used as such.

Several media were used representing those relatively oxidizing as well as those relatively reducing in character. In order to emphasize the significance of this factor in Petri plate work, oxidizing and reducing compounds were added to some of the media. The media, adjusted to pH 6.8, when the experiments were initiated, were as follows:

1. Mannitol nitrate mineral salts
2. Mannitol nitrate plus 0.2 per cent potassium chlorate
3. Mannitol nitrate plus 0.01 per cent potassium permanganate
4. Yeast-extract glucose plus 0.1 per cent cysteine
5. Yeast-extract mannitol plus 0.05 ferrous ammonium sulfate

The ingredients of the basic media are given in the first part of this paper.

Theoretically, the bacteria in a medium that is too much reduced for optimum growth should be stimulated in the region of an oxidizing block, and likewise if the medium in which the organisms are seeded is too oxidizing in character, growth should be enhanced in the vicinity of a reducing block. Further, organisms seeded in a medium that is poised at a potential slightly too high for their optimum growth should be inhibited in the region of a block carrying a greater oxidation intensity. These hypotheses are borne out rather consistently in the experimental data obtained in these studies.

The data in table 2 together with plate 1, give a general picture of the effects of the oxidizing and reducing substances on the growth of these organisms in the various media. The oxidizing blocks all gave distinct stimulation to cell proliferation in media which were somewhat too reduced for the optimum growth of the organisms, for example, in the media reduced with cysteine and ferrous ammonium sulfate (see plate 1, e and f).

TABLE 2

The effect of oxidizing and reducing blocks on the growth of the clover nodule organism (strain 202) in Petri plate cultures

OXIDIZING AND REDUCING BLOCKS	PLATING MEDIA				
	Mannitol nitrate agar	Mannitol nitrate potassium chlorate agar	Mannitol nitrate potassium permanganate agar	Yeast-extract glucose cysteine agar	Yeast-extract mannitol ferrous ammonium sulphate agar
Potassium permanganate 0.07 to 0.1 per cent	−	−	*	+++	+++
Potassium chlorate 0.15 to 0.2 per cent	0	0	*	++	++
Hydrogen peroxide 0.0015 per cent	−	−	*	++	++
Ferric ammonium citrate 1 per cent	+	+	*	+++	+++
Potassium nitrate 0.2 per cent	0	0	*	++	++
Cysteine 0.1 to 0.2 per cent	+++	+++	+++	− to 0	*
Ferrous ammonium sulphate 0.1 per cent	++	++	+++	−	*
Living potato	+++	+++	+++	0 to −	*

+ designates stimulation in the vicinity of the block; 0 growth in plate but no apparent stimulation or inhibition around block; − designates inhibition in vicinity of the block.

* No growth.

The reducing agar blocks (cysteine, ferrous ammonium sulphate, and potato) likewise stimulated growth in media which were too oxidizing in character for the best growth. The mannitol nitrate medium is frequently used in culturing the nodule bacteria, yet the organisms showed well defined growth responses in the vicinity of the reducing blocks.

On the other hand, inhibition of growth was noted around the oxidizing blocks (potassium permanganate) in media (mannitol nitrate, etc.) which at the outset were already too oxidizing for

optimum growth (see plate 1, c). If the media and the blocks possessed practically the same intensity factor of oxidation, no apparent effect was noticed in either direction, as for example a nitrate block in a mannitol nitrate medium. The situation resolves itself into a balancing of the oxidation-reduction intensity factors of the medium on the one hand and those of the blocks on the other.

The behavior of the ferrous ammonium sulfate block in the mannitol nitrate medium is interesting. The block containing 0.1 per cent ferrous ammonium sulphate stimulated growth in this medium in the immediate vicinity of the block, shading off to regions of less and less growth toward the more oxidized areas of the Petri plate (see plate 1, a). By increasing the concentration of the ferrous ammonium sulphate to 0.2 per cent in the block, a zone of inhibition appeared in the immediate vicinity of the block, gradually approaching a region of optimum growth, and again shading off to regions of less growth at the more oxidized side of the Petri plate. (See plate 1, b.) The ring of heavy growth, it is believed, marked a point at which an optimum oxidation-reduction potential was reached for this organism, bordered on the one side by a region too reduced and on the other by a region too oxidized for the best growth of the organism.

An equally interesting behavior of the organisms was observed in the yeast-extract mannitol medium reduced with the ferrous salt and surrounding a potassium permanganate agar block in a Petri plate. It was possible to control the distribution of the growth of the organisms in the uniformly seeded plates by adjusting the balance between the oxidizing intensity of the block on the one hand and the reducing intensity of the medium on the other. When only one-half gram per liter of the ferrous salt was added to the medium, growth was established some distance out into the medium around the permanganate block. By increasing the strength of the ferrous salt from 0.5 to 1 gram per liter, growth appeared only in the immediate vicinity of the permanganate block, often on the very top of the block itself.

The ferric ammonium citrate has been considered an oxidizing agent in these experiments in spite of the citrate radical which it

carries. It behaved, however, as a weak reducing agent in the mannitol nitrate medium as evidenced by a slight stimulation of growth in the vicinity of the block. This growth stimulation may have been due in part to the utilization of the citrate radical by the bacteria as a source of energy. On the other hand, when this block was placed in a highly reduced medium such as yeast-extract mannitol ferrous salts or cysteine agar, a very pronounced growth stimulation was observed as shown in table 2. Again the situation resolves itself into a balancing of the oxidation-reduction intensities of the reacting substances. It would seem that the ferric ammonium citrate is less oxidizing than the potassium nitrate; thus, in a medium containing an appreciable amount of the latter, it may become reducing in nature, while it is oxidizing in the presence of such actively reducing compounds as cysteine or ferrous salts.

Some evidence was obtained in these studies which seems to indicate that the antiseptic properties of certain compounds are due, in part at least, to the poising of the medium at an unfavorable potential for the growth of the organisms. The antiseptic properties of ferrous salts are well recognized, yet by placing these salts in the proper environment, they become distinctly stimulatory in their effect upon bacterial growth. One gram of ferrous ammonium sulphate added to most of these media readily inhibited growth of the organisms. Some of the Petri plate cultures, however, in which growth had been inhibited by the ferrous salts for some ten days, were treated with drops of an appropriate concentration of potassium permanganate, and growth appeared. This indicates that the organisms had previously been unable to initiate growth because of the reducing effect of the ferrous iron. Similar experiments were performed with the potassium permanganate and hydrogen peroxide. It seems that the toxicity of these oxidizing and reducing compounds depends upon their concentrations in relation to the oxidation-reduction character of the environment into which they are introduced.

Similar conclusions have been reached by other workers. Dubos (1929 a) suggested that the toxic action of certain bac-

teriostatic dyes may be due to the "poising" of the media at an unfavorable potential for growth. Methylene blue and the indophenols were found to be no longer bacteriostatic when present in a reduced state in a medium capable of maintaining them in such a condition. Referring to the redox indicators in the study of luminous bacteria, Harvey (1929) suggests that too much indicator (the oxidized form) added to bacterial suspensions will overbalance the reducing capacity of the bacteria and with greater concentrations the luminescence will be permanently quenched. Cannon, Cohen, and Clark (1926) demonstrated that the halogens lose the greater part of their toxicity when added to bacterial suspensions under conditions which prevent the attainment of potentials positive to indophenols.

These experiments clearly emphasize that the numbers of colonies appearing in Petri plate cultures do not necessarily represent all the viable organisms seeded there, even when working with pure cultures. In media poised at a rather unfavorable potential but not completely inhibitory to growth, only a few colonies appeared scattered throughout the plate, but in the vicinity of oxidizing or reducing blocks, which adjusted that immediate area to a more favorable potential, many colonies appeared. By reducing certain areas in the mannitol nitrate medium, for instance, with a cysteine agar block, frequently the colonies appearing in the vicinity of the block were increased twenty to fifty fold as contrasted with the remainder of the plate. It is believed that these few colonies appearing in the media of unfavorable potential originated from clumps of organisms or from individuals possessing an unusually high state of viability. This condition suggests that proper adjustment of the oxidation-reduction character of media used for making quantitative plate counts of these organisms is of importance.

THE INFLUENCE OF THE OXIDATION-REDUCTION CHARACTER OF THE MEDIUM ON PLATE COUNTS

The mannitol nitrate mineral salts agar medium is sometimes used in culturing the nodule bacteria. In the preceding experiments with the oxidizing and reducing blocks in Petri plate cul-

tures, this medium appeared to be somewhat too oxidizing in character for the optimum growth of these organisms. Distinct stimulation was evidenced in the vicinity of the reducing blocks; cysteine, ferrous ammonium sulphate, and living potato blocks. The colonies not only appeared earlier in the region of the reducing ing blocks, but frequently the number of colonies was increased materially as contrasted with the remainder of the plate.

A series of experiments were made to study the effect of various concentrations of cysteine, reducing in nature, on Petri plate counts of the clover nodule organism made with this medium.

The formula for the mannitol nitrate medium is given in the first part of this paper. The cysteine was sterilized in a concentrated solution (0.2 gram in 10 cc. of distilled water) as cysteine hydrochloride, and adjusted to pH 6.8 with dilute sterile NaOH. The media, for the various experiments, were made up at different intervals but were prepared alike in each case. It was necessary also to use different cultures of the organisms as the source of the original inocula in each experiment. No special effort was made to control the age of the organisms or the age of the medium used, except to keep within reasonable limits ordinarily established in laboratory procedure, since in any case, the factors were constant for a given experiment.

Before plating, the medium was liquefied and cooled to 45°C. in a water bath; the cysteine added in proper concentrations, uniformly mixed, and the plates, containing the inocula from the various dilutions, poured. In each experiment an endeavor was made to keep all the conditions involved exactly the same, the only variable being in the amounts of cysteine added. It will be noted that the concentrations of cysteine used in these experiments are much lower than those referred to previously in connection with the cysteine agar blocks and agar shake cultures.

The results of this work indicate that the oxidation-reduction character of the medium is of considerable importance in making Petri plate counts with this organism. The data in table 3 represent some typical results obtained in a few of the experiments carried out. In general, the lowering of the oxidation-reduction potential of the medium by the addition of 0.003 to 0.005 per

cent cysteine gave material increases in the counts, from 2 to 30 times that of the control plates. Above this concentration of cysteine, a gradual decrease in counts below the optimum obtained. In certain experiments, however, media containing concentrations of cysteine as low as .003 per cent gave lower counts than the controls.

The cause for the variations which exist in the effect of similar concentrations of cysteine on the initiation of growth by the bacteria in the various batches of media is not well understood. These variations seem to indicate, however, that the original potentials of the various batches of media, as well as the sensitivity of the organisms to certain potentials at different stages of growth, may vary within certain limits. There is reason to believe that the age of the medium, and the intensity of sterilization, as well as the physiological state of the organisms may be contributing factors. Further studies are in progress to analyze these conditions in more detail.

TABLE 3

The effect of cysteine in mannitol nitrate medium on Petri plate counts of the clover nodule organisms (strain 208)

MANNITOL NITRATE MEDIA PLUS	PLATE COUNTS IN MILLIONS			
	Experiment A*	Experiment B*	Experiment C*	Experiment D*
No cysteine	2.5	10.5	3.5	42.0
0.003 per cent cysteine	21.5	20.5	104.5	21.0
0.005 per cent cysteine	16.5	18.0	89.0	13.0
0.0075 per cent cysteine		15.0		
0.01 per cent cysteine	10.5		79.0	2.0
0.05 per cent cysteine	0.8			

* A new batch of medium and a new culture were used in each experiment.

THE INFLUENCE OF THE OXIDATION-REDUCTION CHARACTER OF THE MEDIUM ON DILUTION COUNTS

Probably one of the most critical stages in the growth of bacteria is the period just following transfer to a new medium. The shock of being introduced suddenly into a new environment and

the necessity of making certain adjustments of, or to, the new environment make the growth initiation period more or less critical. In view of the fact that a liquid medium presents a different set of conditions from those characteristic of solid media, a series of experiments were set up using the liquid manni-

TABLE 4

The effect of the oxidation-reduction character and physical condition of the medium on dilution counts

	INOCULUM CC.						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Clover organism 202—21 days incubation							
Mannitol nitrate medium:							
Check	−	−	−	−	−	−	−
+ 0.002 per cent cysteine	+	+	+	+	+	+	+
+ 0.004 per cent cysteine	+	+	+	+	+	+	+
+ 0.01 per cent cysteine	+	+	+	−	−	−	−
+ reduced iron	+	+	−	−	−	−	−
+ fine sand*	+	+	+	+	+	+	−
+ agar	+	+	+	+	+	+	+
+ ground filter paper*	+	+	+	+	+	−	−
Soybean organism 504—21 days incubation							
Mannitol nitrate medium:							
Check	−	−	−	−	−	−	−
+ 0.002 per cent cysteine	+	+	+	+	+	+	+
+ 0.004 per cent cysteine	+	+	+	+	+	+	+
+ 0.01 per cent cysteine	+	+	+	+	−	−	−
+ reduced iron	+	+	−	−	−	−	−
+ fine sand*	+	+	+	+	+	+	−
+ agar	+	+	+	+	+	+	+
+ ground filter paper*	+	+	+	+	+	+	−

* Lag phase much longer than in other instances, about 5 to 7 days before any of the tubes clouded.

tol nitrate medium and the clover nodule organism. Small experiments finally led to a composite test of several modifications of this medium. The results of a typical experiment are recorded in table 4.

The mannitol nitrate liquid medium was prepared according

to the formula previously given in this paper, with the exception that the agar was omitted. The cysteine was handled in a manner similar to that previously described and added to the medium just prior to seeding the organisms. The reduced iron, the sand, and the powdered agar were autoclaved in a dry condition and added aseptically to the medium in amounts that could be conveniently transferred on the end of a spatula, just before the tubes were inoculated.

In all cases the medium was cooled to room temperature before the different substances were added; this prevented the agar from going in solution. The pulverized filter paper was sterilized directly in the medium.

The organisms for the inocula were taken from a yeast-extract mannitol liquid culture. The various dilutions were made up in 9 cc. water blanks, and a 1 cc. quantity of these dilutions transferred to the various tubes of medium, respectively. Several controls were run to test the sterility of the various substances added to the medium.

One of the striking observations made in this series of experiments was that these organisms which ordinarily initiated growth in the mannitol nitrate agar medium were unable to initiate growth, even in very low dilutions, in the liquid. In dilutions as low as 1 to 10, sufficiently heavy to cloud the medium somewhat, the organisms failed to proliferate, precipitated out of solution, and the medium became clear. On the other hand, by reducing this liquid medium with a very small amount of cysteine (0.002 per cent) growth was secured when the inoculum was reduced to 1 cc. of a 1 to 10 million dilution.

The effect of cysteine on cell proliferation has been studied by several workers and two major explanations of its activities have been offered. First, it has been suggested that the sulfhydryl group (-SH), as such, is a necessary metabolic constituent for cell division; and second, that the reducing effect of the cysteine on the potentials of the medium is responsible for certain stimulatory effects observed in biological systems. Hammett (1929) in his work on mitosis in root tips and the reproduction rate in Paramecium states that "Sulfhydryl is the essential stimulus to

growth by increase in cell number." Quastel and Stephenson (1926) on the other hand, attributed the stimulatory effect of cysteine on the growth of *B. sporogenes* to the reducing action of the cysteine which resulted in a lowering of the potential of the medium, thus creating a more favorable environment for cell multiplication.

Our experimental studies indicate that the oxidation-reduction potentials of the medium are responsible, in part at least, for the behavior of these aerobic organisms in the mannitol nitrate medium, rather than the presence, specifically, of the sulfhydryl group. It will be noted that very similar increases in counts to those obtained by the use of cysteine were induced by the additions of silica sand, filter paper, and plain powdered agar to the tubes, substances which probably are devoid of the -SH group.

As shown in table 4, three general classes of media were used: (1) the untreated mannitol nitrate medium, (2) the medium reduced by varying amounts of cysteine, and (3) the medium to which such chemically inactive substances as filter paper, agar, silica sand, and reduced iron were added. It is believed that the behavior of the organisms under any of these conditions may be interpreted in terms of oxidation-reduction phenomena. No growth was initiated in the untreated medium. This mannitol nitrate liquid medium appears to be poised at a potential too high for growth to occur. Further, due to the convection currents of the liquid, the bacteria were unable to adjust this potential favorably by their metabolic processes. In the second case, the proper concentration of cysteine lowered the potential to a suitable level for the bacteria and cell multiplication proceeded. In the third case, it is probable that the stimulatory effect of such substances as pulverized filter paper, sand, and agar on cell proliferation in this medium was due to the creation of a condition in which the bacteria themselves were able to adjust the environment to conditions favorable for their growth. The reduced iron and possibly the agar may also change the oxidation-reduction potentials. But in view of the diverse nature of the substances used it would seem that the effects were principally due to the

blocking of the convection currents of the medium and the establishment of pockets in the medium sufficiently small and isolated so that the organisms were able themselves to make suitable adjustments of this portion of the medium and proliferate. These findings are consistent with results obtained by Douglas, Fleming, and Colebrook (1917) in their studies on the growth of anaerobic bacilli in fluid media under apparently aerobic conditions by the addition of a number of chemically inactive substances such as charcoal, asbestos wool and the like.

It has been observed by the students in this field that cells of microörganisms in some way assist each other in growth and can adjust their environment within certain limits to conditions more favorable for growth. Gillespie (1913) found that larger inocula of pneumococci were required to start growth in broth than on an agar medium, and concluded that organisms in some way assist each other in growth when they are closely associated while when they become scattered in a liquid medium, this favoring action is lost. To a somewhat similar condition Churchman and Morton (1921) applied the term, "communal activity" and Robertson (1922) the term, "allelocatalysis." Clark (1924) states that actively growing anaerobes, if not overtaxed, can establish their own reducing conditions even in the presence of a certain amount of molecular oxygen. Webster (1925), Burnett (1925), Quastel and Stephenson (1926), and Dubos (1929b) all working under different conditions with different organisms have come to the conclusion, that certain media while capable of growing the organisms when heavily seeded do not allow isolated individuals or even small inocula to develop.

The organic nitrogen carried by the cysteine may be possibly a contributing factor to the stimulatory effect of this compound on the growth of these organisms. Increasing the cysteine, however, from 0.002 to 0.01 per cent caused a depressing action (table 4). If it were wholly a question of a more available nitrogen source, it is hardly conceivable that such a small increase should exhibit a material depressing effect on cell multiplication.

Moreover, the increases in growth produced by the addition of such rather inactive substances chemically as filter paper, sand,

and reduced iron can hardly be accounted for on this basis. Other data, not recorded in this paper, obtained on the stimulatory effect of cysteine on these organisms in an aged yeast-extract mannitol medium, which carries organic nitrogen, seem to emphasize the importance of another factor aside from a purely nutritional requirement.

It is difficult to attribute the entire effect of cysteine on bacterial growth under these conditions to any one specific cause, yet there seems to be sufficient evidence to indicate that the reducing action of cysteine is at least one of the contributing factors responsible for the increase in cell proliferation observed in these studies.

SUMMARY

These studies indicate that the oxidation-reduction character of bacteriological media exerts an important influence on the growth and behavior toward atmospheric oxygen of certain aerobic bacteria (Rhizobia).

In agar shake cultures these organisms establish thin zones of growth, the level of which depends on the oxidation-reduction character of the substrate.

The ability of these organisms to initiate growth is determined in part by the oxidation-reduction character of the medium. By oxidizing and reducing certain restricted areas in Petri plate cultures, marked contrasts were observed in the number of colonies appearing on the treated and untreated portions. By reducing with appropriate concentrations of cysteine a medium which is slightly too oxidized for the best growth of these organisms, it is possible to increase greatly the number of sources which develop into colonies in Petri plate cultures or to lessen the size of inoculum necessary to secure growth in tubes of liquid media. It is evident that the oxidation-reduction character of the medium is of importance in making counts of these organisms by either the Petri plate or dilution methods.

These organisms are able to make certain adjustments in the oxidation-reduction character of media which are slightly too oxidized for their growth. The introduction of sand, finely

divided filter paper, etc. into a liquid medium, which was too highly oxidized to permit growth, provided conditions which allowed the organisms to develop. Apparently this was due to the trapping of small volumes of the medium where the organisms were able to make the necessary adjustments. Development in such tubes was slower than in tubes in which the medium was reduced with appropriate concentrations of cysteine. The lengthened lag phase seems to represent the time occupied by the organisms in changing the potential of the medium to a more favorable condition.

The bacteriostatic effect of certain oxidizing and reducing substances seems to be due in part to the poising of the medium at an unfavorable potential for growth. The toxicity of these compounds depends largely upon their concentration in relation to the oxidation-reduction character of the system in which they are introduced. Similar concentrations of the same compounds were found inhibitory to bacterial growth under one set of conditions and stimulatory under another.

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PLATE I

THE EFFECT OF OXIDIZING AND REDUCING BLOCKS ON THE GROWTH OF RHIZOBIUM (CLOVER 202) IN PETRI PLATE CULTURES

a. Mannitol nitrate medium, with a 0.1 per cent ferrous ammonium sulfate block.

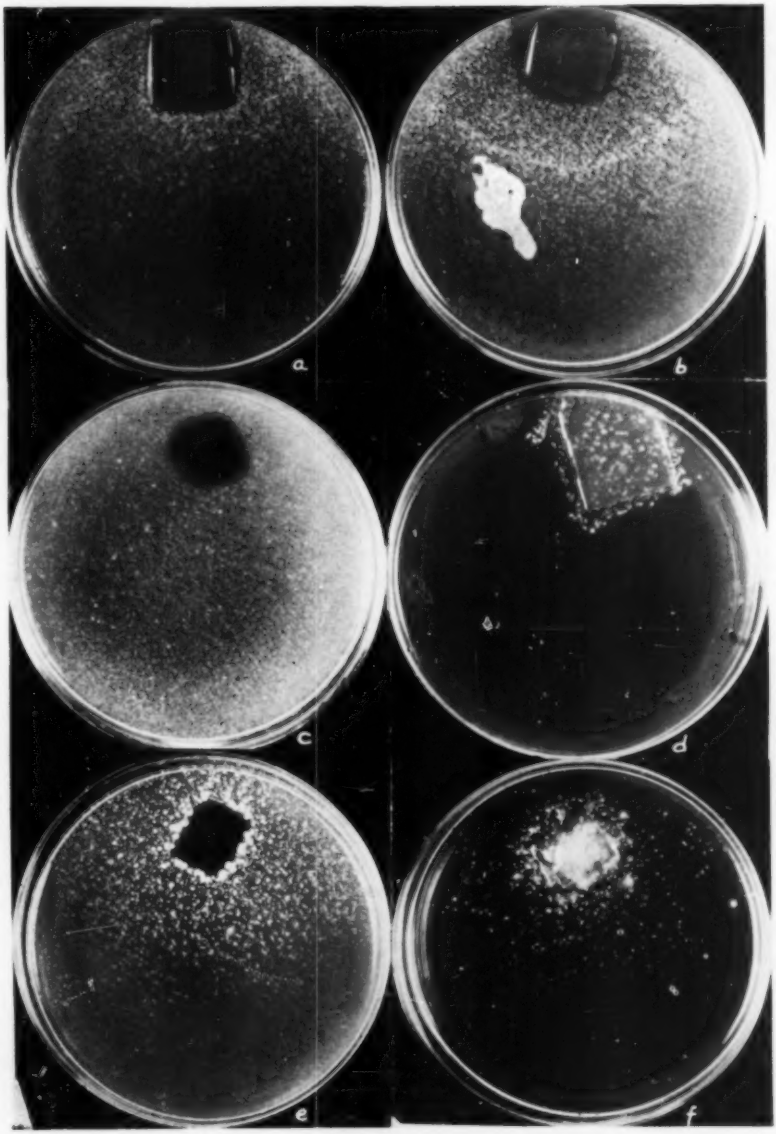
b. Mannitol nitrate medium, with a 0.2 per cent ferrous ammonium sulfate block.

c. Mannitol nitrate medium, with a potassium permanganate block.

d. Mannitol nitrate, potassium permanganate medium with a cysteine block.

e. Mannitol ferrous ammonium sulfate medium with a potassium permanganate block.

f. Glucose cysteine medium with a potassium permanganate block.



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